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Induction of mucosal immune responses in the horse

Richard Easeman BSc (Hons)

**AWARDING BODY:
THE OPEN UNIVERSITY**

A thesis submitted in candidature for the
degree of Doctor of Philosophy

March 1997

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Animal Health Trust

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Preface

The work reported here was carried out in the laboratories of the Centre for Preventive Medicine, Animal Health Trust. The studies represent the work carried out by Richard Easeman, unless otherwise stated in the text. This dissertation has not been submitted as whole or in part to any other university.

I would like to thank my fiancée Annie and our families, this thesis is dedicated to all of them, present and past.

Acknowledgments

The work described in this thesis was carried out in the laboratories of the Center for Preventive Medicine at the Animal Health Trust. I would like to thank, therefore, Dr. Jenny Mumford, Head of the Center for Preventive Medicine, for the use of the laboratory facilities. A great part of the work described herein was generously funded by the Home of Rest for Horses.

I would like to acknowledge the help and support given to me by all the laboratory and estate staff during this study. I acknowledge the helpful discussions that I have had with Dr. Mark Roberts concerning the ELISPOT assay. My warm thanks must go to Dr. Tony Blunden for the electron-micrographs of equine BALT, Dr. Matthew Breen for help with graphics, and Miss Zoe Swann for her lively encouragement and help in growing up the stocks of monoclonal antibodies. I would also like to thank my supervisors, namely Dr. Duncan Hannant, Prof. Chris Stokes, and Dr. Jenny Mumford for their thoughtful guidance. My biggest thankyou must go to those who were directly responsible for my scientific training, Dr. Duncan Hannant for his patient and invaluable supervision, Dr. Julia Kydd and Dr. Terry O'Neill for their helpful hints and discussions.

I would like to thank all persons who gave up their time to vaccinate, sample, and wrestle the ponies on my behalf, these include: Duncan Hannant, David Jessett, Julia Kydd, Terry O'Neill, Neil Chanter, Javier Castillo-Olivares and Richard Newton.

Induction of mucosal immune responses in the horse

Abstract

The studies described in this thesis concern the examination of the equine mucosal immune system and the investigation of the efficacy of mucosal vaccination for protection of horses against the common respiratory pathogen, equine influenza virus (H3N8).

Stimulation of immune responses at the nasopharyngeal induction site was measured by detection and enumeration of circulating antigen-specific antibody-secreting cells (ASC) in the peripheral blood. Cholera toxin B chain (CTB) given intranasally induced CTB-specific antibodies in both serum and nasal washes (mucosal effector site) which was preceded by the appearance of CTB-specific ASC in the peripheral blood. This confirmed the function of a local immune system in the respiratory tract of the horse, and suggested the existence of a common mucosal immune system in horses.

Techniques were developed to measure neutralising antibody in nasal secretions of ponies after infection with influenza virus (strain A/equi-2/Sussex 89) and intranasal vaccination with inactivated virus and CTB. The vaccination protocol, using systemically primed animals, elicited circulating influenza-specific ASCs, serum haemagglutination-inhibiting antibodies and nasopharyngeal virus neutralising antibodies. Intranasally vaccinated ponies were completely protected from challenge infection with influenza virus of the homologous strain. This has been the first demonstration of the efficacy of intranasal vaccination using inactivated influenza virus in horses.

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Chapter 1

Introduction

1.1 Mucosal Immunity

1.1.1 Introduction

The mucosal epithelium forms the barrier between the external and internal environments. Its surface area is enormous, in man it has been estimated at around 400 m² (McGhee and Kiyono, 1993) while the equine lung alone is around 2000 m² (McGorum, 1995). The nature of the mucosal tissues brings them into daily contact with large quantities of respired and ingested antigens, including viruses and bacteria. This interface is therefore the major route of infection for respiratory and gastrointestinal pathogens. Consequently immunological effector mechanisms working at the level of the mucosal surface are very important.

Experimental investigations in a wide variety of species, including mouse and man, have demonstrated the existence of the common mucosal immune system (CMIS). The CMIS functions largely as a separate entity from the systemic immune system, protecting the mucosae from infection using combinations of humoral, mainly dimeric secretory IgA (sIgA), and cellular mechanisms, including phagocytic cells and cytotoxic T lymphocytes (CTL). The great importance of the CMIS for protection from mucosal pathogens, is indicated by the metabolic effort expended in maintaining it. It has been estimated that about 75% of all plasma cells in the human body are committed to the production of sIgA in mucosal tissues (Phillips-Quagliata and Lamm, 1994), which equates to almost 5 grams of antibody production per day (McGhee and Kiyono, 1993).

During the stimulation of a mucosal immune response, antigen is first taken up, processed, and presented to T and B lymphocytes at the site of deposition or local lymph nodes (the induction site). Lymphocytes become activated within the induction site and migrate

through the blood to distinct effector sites where they terminally differentiate, under the influence of antigen and cytokines, to become antibody producing plasma cells and effector T lymphocytes (figure 1.1). Thus an immune response stimulated at one site can result in an effector immune response in another, for example vaccination in the gut can elicit antibody production in the lung (McGhee and Kiyono, 1993).

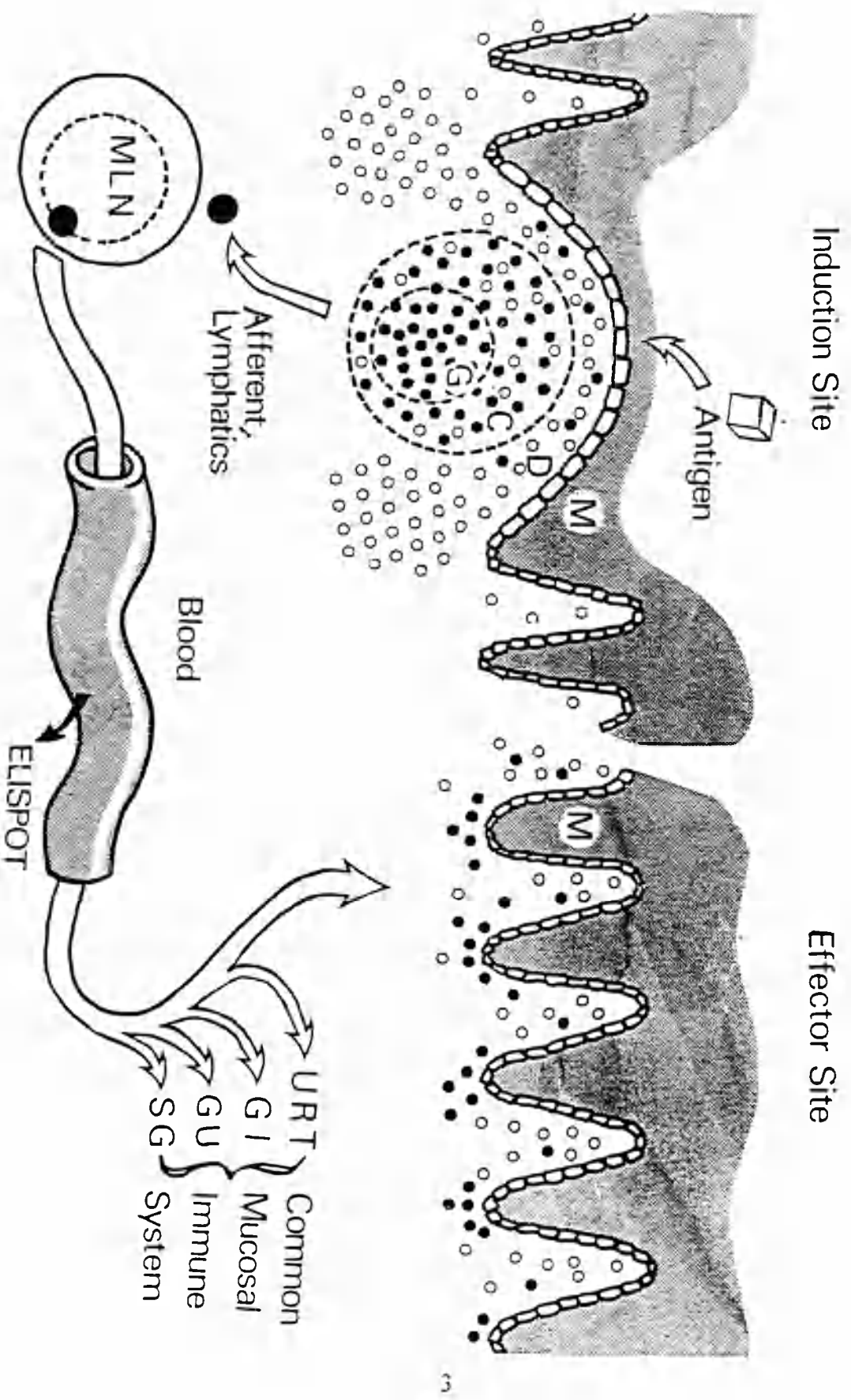
1.1.2 Induction sites

The most commonly studied induction site for mucosal immune responses is the gut associated lymphoid tissue (GALT). GALT is a general term for lymphoid tissues distributed in the intestine, including aggregated lymphoid nodules called Peyer's patches (PP's, Griebel and Hein, 1996). These "raised areas" in the intestinal mucosa were first described by Johanni Conradi Peyer in 1677 (Croitoru and Bienenstock, 1994 (figure 1.1)), although it was not until the mid nineteenth century that they were implicated as a lymphoid tissues (Brucke (1851), as cited by Croitoru and Bienenstock, 1994). During the 1970's PP's were more precisely defined in the rabbit where an enriched population of IgA precursors was identified that seeded into other areas of the gut (Craig and Cebra, 1971). Cell-migration studies in mice showed that PP's were a source of antigen specific IgA producing cells which migrated to other regions of the gut lamina propria (Husband and Gowans, 1978).

Specialised antigen transporting cells (M cells) were described as the microfold epithelial structures overlying human PP's (Owen and Jones, 1978). The function of these M cells is to transport antigens, viruses, bacteria and protozoa from the gut lumen to the underlying lymphoid cells. The follicle-associated epithelium has specialised structural differences compared with the surrounding villus epithelium which allows greater uptake of antigen. These include a reduced number of goblet cells, a much reduced mucus layer, and a lack of secretory component expression (poly Ig receptor-see below) and IgA secretion.

Figure 1.1 The Common Mucosal Immune System (CMIS).

Figure 1.1 shows a schematic representation of the induction of a mucosal immune response in a Peyer's patch (PP). An antigen in the lumen passes through the mucus (M), is taken up by the specialised lymphoepithelium of the PP, and is processed and presented to B (closed circles) and T (open circles) cells. The stimulated B-lymphocytes exit the PP *via* afferent lymphatics, traffic through the blood, and repopulate distant mucosal effector sites. Trafficking B-lymphocytes can be detected using an ELISPOT technique. Abbreviations: M, mucus; D, dome; C, cortex; G, germinal centre; MLN, mesenteric lymph node; URT, upper respiratory tract; GI, gastrointestinal tract; GU, genitourinary tract; SG, secretory glands.



Within the PP of rabbits, B cells are localised in the dome area and germinal centres (Ermak *et al.*, 1994, Phillips-Quagliata and Lamm, 1994). T cells are present in these areas but also in distinct T-cell areas between the follicles. About 80 % of PP T cells are CD4 +ve helper T cells, and about 20% are CD8 +ve cytotoxic/suppressor cells (Ermak *et al.*, 1994).

The dome in rabbit PP's, beneath the follicle epithelium, is characterised by CD4 +ve helper T cells, dendritic cells, macrophages, and IgM +ve B cells (Ermak *et al.*, 1994, Phillips-Quagliata and Lamm, 1994). In other species, such as the mouse and human, PP dome areas may contain only very few B cells. CD8 +ve T cells are rare in both GALT domes and germinal centres. The germinal centres contain isotype-switched B cells, CD4 +ve helper cells, follicular dendritic cells and macrophages that are phenotypically distinct from those found in the dome area (Ermak *et al.*, 1994).

Similar mucosal lymphoid follicles are found elsewhere in the CMIS, such as the nasopharyngeal associated lymphoid tissue (NALT; Pabst and Tschernig, 1997), the bronchae (BALT; Sminia *et al.*, 1989), and the genitourinary system. The NALT, principally the Waldeyer's ring in humans, has great structural and functional similarities to GALT. An extensive collection of reviews concerning the structure of NALT, and its use in intranasal vaccination strategies, has recently been published (Pabst and Tschernig, 1997; Brandtzaeg and Haneberg, 1997; Tamura and Kurata, 1997; McDermott and Snider, 1997; Russell and Wu, 1997; Kyd and Dunkley, 1997; and Staats, 1997). The role of NALT in the induction of a secretory immunoglobulin response is supported by the finding that tonsillectomy and adenoidectomy patients have reduced amounts of IgA in the nasopharynx (Donovan and Soothill, 1973) and peripheral blood (Del Rio-Navarro *et al.*, 1995). The nature of the immunological response by NALT depends on the physicochemical state of the antigen to which it is exposed. Soluble antigen is taken up

by epithelial cells and macrophages, and in general, this establishes either tolerance or a systemic immune response depending upon the immunological properties of the antigen itself. Particulate antigens are either removed by mucocilliary clearance or taken up by M cells and reach the NALT, particularly in conditions where the mucocilliary escalator is damaged, for example during an infection with respiratory virus..

1.1.3 Effector sites

B Lymphocyte responses

The effector arm of the CMIS is made up of a combination of effector T and natural killer (NK) cells, and secretory immunoglobulin, of which IgA dominates. In the upper respiratory tract (URT), salivary and mammary glands, gastrointestinal and genitourinary systems, IgA is produced locally. It has been calculated that approximately 5×10^{10} IgA producing plasma cells populate the human small intestine, compared with 2.5×10^{10} in the spleen and lymph nodes, making the gut the most important area for antibody production in the body.

B cells, once stimulated in the induction sites, GALT or BALT, proliferate and become switched to IgA production within the nodule (Jones and Cebra, 1974). They pass into the regional lymph node, e.g. mesenteric lymph node, continue to proliferate and mature into blast cells capable of migration (Gowans and Knight, 1964). The cells enter the blood stream via the efferent lymphatics and selectively locate to the lamina propria (LP) due to the interaction of specific "homing antigens" on the surface of the lymphoblasts (Jalkanen *et al.*, 1986) and specific receptors, "addressins", on the surfaces of the high endothelial venules (Streeter *et al.*, 1988) (see chapter 3). The B cells may locate to specific areas of

the LP, for example in the pig where antibody positive cells are 10 times more numerous in the crypts than in any other area in the LP (Vega-Lopez *et al.*, 1993).

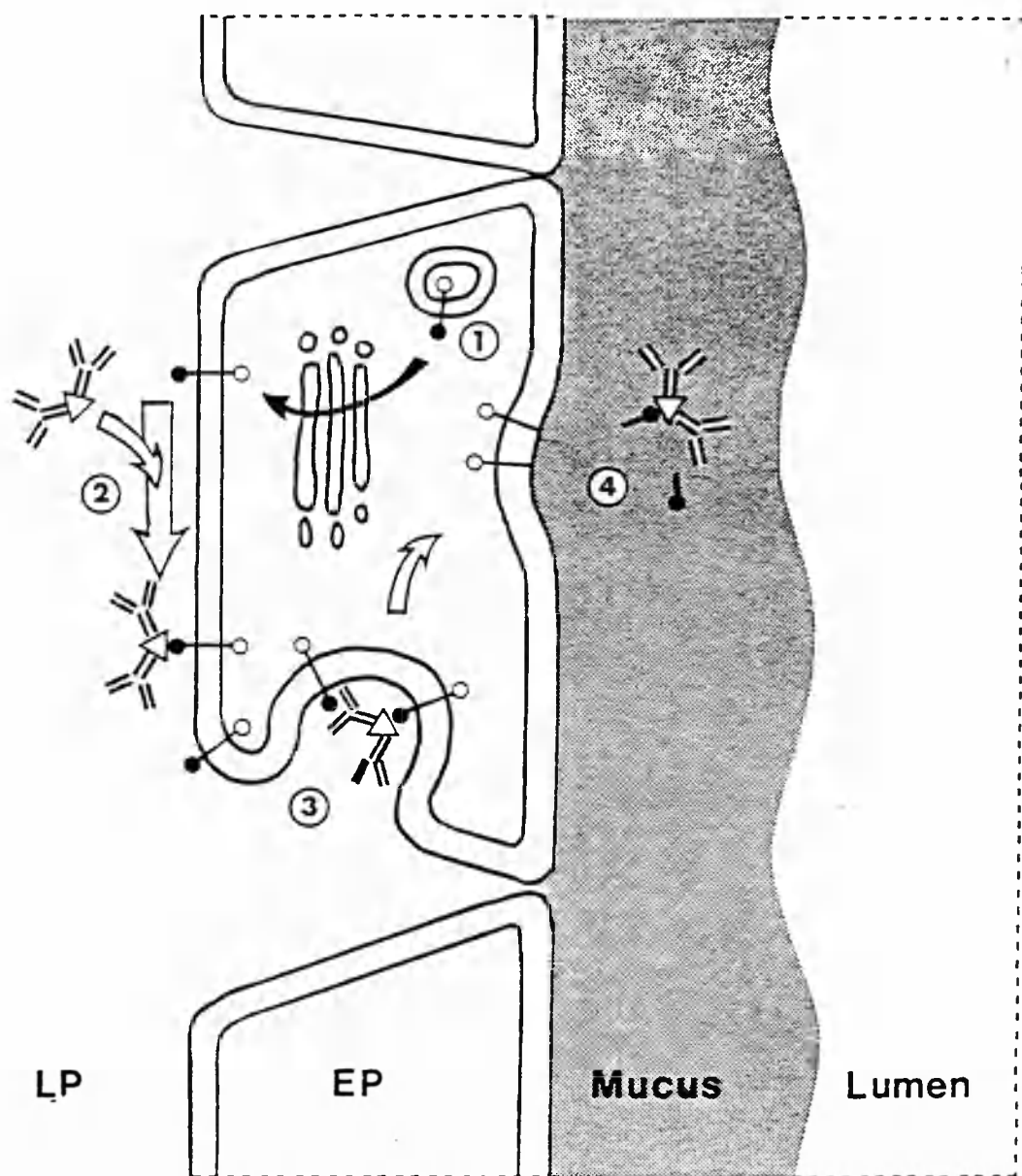
Human IgA occurs as two subclasses (IgA1 and IgA2) and may be differentially distributed in serum and external secretions. Human serum contains mainly monomeric IgA1, whereas dimeric IgA1 and IgA2 are found in human secretions (McGhee and Kiyono, 1993). The two immunoglobulins share a 95% homology in amino acid sequence, however, differences around the hinge region of IgA2 confers an increased resistance to proteolytic enzymes (Mestecky and McGhee, 1987). The distribution of IgA1 and IgA2 plasma cells in different human mucosal effector sites suggests that different induction sites may contribute differentially in the provision of B cells. For example the LP in the URT of humans consists mainly of IgA1 producing plasma cells, with very few IgA2 cells. In contrast IgA2 plasma cells dominate in the LP of the lower intestine. Similarly, if B cells from tonsillar lymphoid tissues are stimulated in tissue culture they produce mainly IgA1 (>95%). Appendix B cells produce both IgA1 and IgA2. This suggests that stimulation of the CMIS in the URT of humans preferentially populates the URT with IgA1 producing cells, while stimulation of the PP populates the gut with IgA2 producing cells (McGhee and Kiyono, 1993).

The B cell effector sites, the lamina propria of the URT, gut, or genitourinary system, are covered by epithelial cells that express the polymeric Ig receptor (pIgR) also known as secretory component (figure 1.2). The polymeric immunoglobulins produced in the LP bind to the pIgR and are then transported through the epithelial cell and are released into the external secretions (Kaetzel *et al.*, 1991).

Figure 1.2 Secretion of IgA into mucus.

Figure 1.2 shows a schematic representation of the secretion of dimeric IgA from the lamina propria (LP), through the epithelium (EP), into the mucus.

1. Synthesis of the secretory component (SC) in the rough endoplasmic reticulum.
Phosphorylation and glycosylation in the golgi apparatus.
2. Binding of extracellular IgA to form SC-IgA.
3. Endocytosis and transcytosis of SC-IgA complex.
4. Release of SC-IgA and free SC into the lumen.



T lymphocyte responses

T cells also play a major role in the recovery from mucosal infections and may also ameliorate their outcome. In the gut, two populations of T cells can be identified, intraepithelial (IEL) and lamina propria (LPL) lymphocytes.

IEL are located in the murine intestinal epithelium, between the enterocytes, separated from the gut lumen by tight junctions and from the lamina propria by the basement membrane. In man IEL can comprise up to 20 % of the total cells in the epithelium (Dobbins *et al.*, 1986). IEL represent a complex mix of T lymphocytes which are distinguished from peripheral T lymphocytes by the differential expression of cell surface antigens, which may reflect differences in their function (for review see LeFrancois, 1994). For example while IEL's express T cell receptors (TcR) comprised of both $\alpha\beta$ and $\gamma\delta$ chains, they express a much larger proportion of $\gamma\delta$ chains than are found in other lymphoid organs (Deusch *et al.*, 1991). IEL's characteristically exhibit a larger proportion of CD8 +ve cells than are found in other T cell types, with up to 80% of TcR $\gamma\delta$ IEL being CD8+ve compared with approximately 25% in the peripheral blood (Jarry *et al.*, 1990). Human and murine $\gamma\delta$ IEL, express CD8 in the homodimeric $\alpha\alpha$ form rather than the more common $\alpha\beta$ heterodimer.

Studies into the origin of these cells have shown that a large proportion of the $\gamma\delta$ IEL are extrathymically derived and maturation can be observed at this site during embryogenesis (Carding *et al.*, 1990; Guy-Grand *et al.*, 1991). IEL's expressing the $\alpha\beta$ TcR are mostly thymus dependent (Rocha *et al.*, 1991). A subset of CD8 $\alpha\alpha$, TcR $\alpha\beta$, however, utilise TcR "forbidden" V β genes not found in peripheral T cells, and therefore may also be extrathymically derived (Rocha *et al.*, 1991; LeFrancois, 1994).

The majority of human CD3 +ve IEL express CD45RO, indicating that they are memory or recently activated cells (Halstensen *et al.*, 1990). This is supported by the observation that the proportion of porcine IEL that express IL-2R is greater than that seen in peripheral blood (20% compared with 7% respectively; Bailey *et al.*, 1992). Murine IEL have been shown to be directly cytotoxic upon isolation, unlike peripheral CD8 +ve T-cells which usually require to be stimulated *in vitro* before acquiring cytotoxic activity. This suggests that IEL are activated cells maintained in a state ready to kill potential targets (Guy-Grand *et al.*, 1991).

In the human, approximately 50 % of LP T cells are CD4 +ve (the "helper" phenotype) and 35 % express CD8 and are of the cytotoxic/suppressor phenotype. The majority of these cells appear to be memory cells as they are CD45RO +ve (Zeitz *et al.*, 1991). There is a similar situation in mice where greater than 50 % of all gut LP T cells express CD4 (Mega *et al.*, 1991). It has been suggested that the function of human and mouse LPL is to provide help for the B cells present in the LP (Kanoff *et al.*, 1988; Mega *et al.*, 1991). T cell subsets are spatially segregated within the LP and in the adult porcine gut for example, LP CD4 +ve cells are largely restricted to the core of the villus. CD8 +ve cells, in contrast, line the external margins of the LP core, in contact with the basement membrane of the epithelium (Vega-Lopez *et al.*, 1993).

1.1.4 Antigen uptake and presentation.

There are three major routes for antigen to cross the epithelium, (i) antigen may cross the epithelium paracellularly, that is, between the epithelial cells over the tight junctions, (ii) antigen may pass intracellularly by means of cell absorption, and (iii) antigen may be taken up and actively transported to lymphoid follicles such as BALT or GALT.

M cells are a specialised epithelial cell devoted to the transport of luminal antigens and they exhibit short microvilli, apical vesicles and an intracellular vesicle transport system (see below, Bye *et al.*, 1984). The PP renews its epithelium every 3-4 days (Smith *et al.*, 1980) and hence, M cells have to be regularly replenished from the crypts surrounding the base of each dome.

M cells in the PP of the rabbit are located close to the tip of the dome, have large pockets in their basolateral membrane containing B lymphocytes and CD4 and CD8 double negative T cells. Tracer particle experiments have shown selective transport across the M cell to these basolateral pockets. A large range of particle sizes may be transported, from macromolecular complexes such as ferritin (<10 nm), virus particles and liposomes (20-200 nm) and large microspheres (0.5-10 μ m). However, there is evidence that smaller particles (in the nanometer diameter range) may be more efficiently taken up than those of a larger size (in the micrometer diameter range) (Maloy *et al.*, 1994; O'Hagan, D. personal communication). Uptake of synthetic particles into M cells is extremely rapid, with molecules appearing at the basolateral surface as soon as 30 minutes after introduction into the lumen. Large particles (>5 μ m) are transported to, and remain within, the lymphoid follicle whereas smaller particles (<5 μ m) are transported through the PP to mesenteric lymph nodes and peripheral lymphoid organs (Ermak *et al.*, 1994).

The role of M cells in processing and presentation of antigen is unclear, although they can express MHC class II molecules and possess the lysosomal compartments required for processing (Allen *et al.*, 1993). M cells have also been shown to stimulate T cells *in vitro* and secrete the cytokine IL-1 (Pappo and Mahlman, 1993). However, although CD4 +ve T cells are not found in close proximity with M cells, CD8 +ve T cells are found in the M cell pockets where they could recognise M-cell processed antigen (Ermak *et al.*, 1994;

Jarry *et al.*, 1990). Other professional antigen presenting cells (APC) such as dendritic cells and macrophages extend their processes into the M cell pocket and probably co-operate with the M cell in antigen processing and lymphocyte stimulation. However, the division of labour is unknown (Kato and Owen, 1994).

Dendritic cells (DC) are widely distributed in the normal lung and gastrointestinal tracts (Holt *et al.*, 1990 and 1994). These mobile leukocytes are characterised phenotypically by long dendritic processes, irregular nuclei, and the small numbers of phagolysosomes. Although they do not have a prominent phagolysosomal system, they are actively macropinocytic and have numerous endosomes and appropriate lysosomal enzymes. They lack the phenotypic markers for mononuclear phagocytes and constitutively express high levels of MHC class II (for review see Toews, 1991). DC's are reported to play an extremely important role in the induction of immune responses after a respiratory infection. Very quickly after the onset of both bacterial and viral infections DC's are observed to recruit in large numbers to the site of infection (McWilliam *et al.*, 1994 and 1997) and then traffic to local lymph nodes to present the processed antigen to naive T-cells (Hamilton-Easton and Eichelberger, 1995).

Lung DC's are excellent accessory cells for naive or primed T cells and are 10 to 100 times more potent than alveolar macrophages (AM) in inducing T cell proliferation (Kradin *et al.*, 1991; Havenith *et al.*, 1993; Rochester *et al.*, 1988). DC's are found in large numbers in close association with the follicle epithelium and are known to take up antigen from the gut and airway lumens. For example, antigen injected into the gut has been detected in the afferent lymphatics in association with dendritic cells (Liu and Macpherson, 1991). Although these cells were also shown to present this antigen to primed T cells, the site of presentation *in vivo* remains unknown. Xia *et al.* (1995) also demonstrated that intra-tracheal administration of hen egg lysozyme (HEL) resulted in

DC's which were capable of stimulating the proliferation of primed T-cells. These antigen presenting DC's were found both in the lung, from 3 hours to 7 days after administration, and in the regional lymph node from 24 hours after administration. Confirmation that DC's could induce T cell priming *in vivo* was obtained by Havenith *et al.* (1993) who showed that very soon after adoptive transfer into the trachea of rats of *in vitro* antigen-pulsed DC's, antigen specific T cells appeared in the local lymph nodes. This effect could be reproduced using adoptive transfer of antigen-pulsed alveolar macrophages (AM) but 10 times more cells were required to induce the equivalent response.

The important role of DC's as "professional" APC's has been emphasised by the observation that they can activate MHC class I restricted, CD8+ve antigen specific CTL *in vitro* even in the absence of CD4+ T-cell help (Inaba *et al.*, 1987; Boog *et al.*, 1988).

Macrophages are also present along the entire respiratory tract, including the airway lumen, the lamina propria, and the lymphoid follicles. They are efficient phagocytic cells, internalise large quantities of foreign antigens and process it for presentation by MHC class II molecules on the cell surface. Porcine alveolar macrophages have also been shown to express IL-1 and TNF- α (Chitko-McKown *et al.*, 1991). The capacity of AM to activate naive lymphocytes appears to be weak (Hance, 1991) and their function has often been proposed to be in the inhibition of potentially harmful inflammatory immune responses (for review see Holt, 1986), so much so that depletion of AM's *in vivo* enhances the capacity of experimental animals to mount an immune response to inhaled antigens (Thepen *et al.*, 1989). This down-regulation of lung T-cell responses seems to function by preventing T-cell responsiveness to IL-2, but still permitting T-cell activation and cytokine secretion, as measured by up-regulation of IL-2R expression and IL-2 production (Upham *et al.*, 1995). The AM-suppressed T-cells become locked in G₀/G₁ phase, through a failure to transduce IL-2 signals leading to phosphorylation of IL-2R-associated

proteins, and are unable to proliferate upon stimulation with Con A (Strickland *et al.*, 1996).

It is thought that once an inflammatory response is underway within the lung the role of the AM may change. Under the influence of inflammatory cytokines and infiltrating activated T cells, which AM's are capable of stimulating, AM's may switch from their normally suppressive role to one of activation of the immune response (Hance, 1991).

The epithelial cells of the URT also may play a large role in antigen presentation. There is evidence for accessory cell function by human MHC class II expressing nasal turbinate and bronchial epithelial cells (Kalb *et al.*, 1991). Intestinal epithelial cells are capable of activating CD4 +ve T cells (Kaiserlain *et al.*, 1989) but to a lesser extent than CD8 +ve T cells (Bland and Warren, 1986). Under most circumstances the "suppressor phenotype" would appear to prevail, but in inflammatory conditions CD4+ve cells may be preferentially activated (Panja and Mayer, 1991).

1.1.5 Cytokine regulation of the mucosal immune system.

When considering the cytokine regulation of the mucosal immune system it is important to remember the distinction between mucosal induction and effector sites. It is often convenient to consider cytokine environments in terms of Th1 and Th2 as described in murine CD4 +ve T cell clones by Mosmann and Coffman (1989). However, it should be noted that such clear partitioning has not been observed in "whole T-cell" populations or in other species. Further, CD8 +ve T cells, APC's, and M cells also secrete cytokines which may complicate interpretation of different cytokine profiles within different microenvironments. Traditionally, Th1 cells are associated with cell mediated effector mechanisms such as cytotoxic lymphocytes (CTL) and can be characterised by the

production of INF- γ and IL-2. Other cytokines often directly associated with the induction of Th1-type immune responses are not produced by T-cells, such as IL-12. In contrast, Th2 cells are associated with humoral responses and the secretion of IL-4, IL-5, IL-6, IL-10 and tumour necrosis factor- β (TNF- β).

Induction sites

In the CMIS, the induction of an immune response occurs at the level of the mucosal epithelium, which areas might primarily include the mucosae associated lymphoid tissue (MALT) excluding the germinal centres, and involves the uptake, processing and presentation of antigen to T cells, B-cell stimulation and isotype switching, followed by clonal expansion of antigen specific B cells.

APC's are also a major source of cytokines. After antigen uptake, M cells have been shown to secrete IL-1, an activator of T cells (Pappo and Mahlman, 1993). Cytokines are also pivotal in the induction of an immune response by stimulating traffic of APC's to the lymphoid tissues. Interferon- γ (IFN- γ) has been shown to selectively increase the numbers of DC's found in the lung (Kradin *et al.*, 1991). MacPherson and colleagues (1995) implicated TNF- α in the migration of DC's from the intestine towards the draining lymph nodes, thereby suggesting that TNF- α is important in regulating antigen presentation. Pig lung macrophages may also play a role in this regulation by producing TNF- α and IL-1 upon activation with antigen (Chitko-McKown *et al.* 1991). Once present in the local lymph nodes, the presentation of DC associated antigen is up-regulated by granulocyte monocyte colony stimulating factor (GM-CSF, Cumberbatch *et al.*, 1991). Another mechanism by which cytokines might enhance antigen presentation is the upregulation of MHC class II expression on epithelial cells by IFN- γ (Steiniger *et al.*, 1989) derived from intraepithelial CD8 +ve T cells (Cerf-Bensussan *et al.*, 1984).

In mice, the induction of IgA B cell responses requires contact with a T cell membrane and is IL-4 and transforming growth factor β (TGF- β) dependent. IL-4 and TGF- β promote isotype switching to IgA (Husband *et al.*, 1994), whereas this is inhibited by INF- γ , underlining the importance of separate microenvironments within the CMIS (Hodgkin *et al.*, 1991). Subsequent differentiation and proliferation is also cytokine dependent, in that IL-5, 6 and 10 induce proliferation and amplify IgA secretion, and is independent of T cell contact (Husband *et al.*, 1994). The cells responsible for production of these cytokines in PP's can be CD4 +ve, expressing IL-2,4,5,6 and INF- γ (Hiroi *et al.*, 1994 and 1995), or CD8 +ve T cells expressing IL-4, 5, 10 and INF- γ (Lagoo *et al.*, 1994).

Effector sites

Mucosal effector sites are populated by CD4 +ve and CD8 +ve T cells as well as large numbers of IgA producing plasma cells. As such, there are a large number of cytokine requirements to service the different effector functions. This can be partly explained by the spatial distribution of cytokine secreting cells. For example, LP cells expressing mRNA for INF- γ are restricted to the basal regions, whereas IL-4, 5, and 6 are found throughout the LP and villi (Husband *et al.*, 1994). It is interesting to note that in this study IL-6 mRNA was never found in PP's. Work by Taguchi *et al.* (1990) showed that IL-5 secreting cells outnumbered INF- γ secreting cells by 3:1 in the LP whereas the ratio in the PP was approximately 1:1. Therefore, they concluded that IgA effector but not IgA inductive sites were Th2 dependent. This may be somewhat of an oversimplification given that Th2-type cytokines such as IL-4 and TGF- β are required for IgA isotype-switching at the induction site. Another illustration of the requirement for spatial segregation of

cytokine production is demonstrated by INF- γ , which although not traditionally associated with Th2 type cytokines, is produced by IEL's (Cerf-Bensussan *et al.*, 1984) and is required for epithelial cell pIg receptor expression (Youngman *et al.*, 1994).

Experiments with IL-4 and IL-6 knockout mice showed the differential requirement for these cytokines in the induction and maintainance of IgA responses. IL-4 knockout mice were deplete of IgA plasma cells and failed to mount an IgA response to antigens injected into the PP (Husband *et al.*, 1994). In IL-6 deficient mice, only small numbers of IgA secreting plasma cells were seen in the intestine compared with controls. This translated into a reduction in influenza specific IgA responses following inoculation with HA-expressing vaccinia virus, whereas normal IgA responses could be restored in these mice by infection with a recombinant vaccinia virus also expressing the murine IL-6 gene (Ramsay *et al.*, 1994). Recently, Bromander and colleagues (1996) have published seemingly contradictory evidence in which IL-6 deficient mice exhibited normal mucosal IgA responses against OVA. This dichotomy may be explained by the different forms of antigen used in these experiments, or differences in the MHC haplotype of the mouse strains.

As in the induction sites, cytokines in the effector sites may be secreted by CD4 +ve or CD8 +ve T cells. For example, freshly isolated CD8 +ve IEL secrete cytokines normally associated with both Th1 and Th2 helper function, including IL-5 and INF- γ (Taguchi *et al.*, 1991)

1.1.6 Immunology of the equine respiratory tract.

Equine URT architecture has been little investigated, and studies by Mair (1987 and 1988) form the large part of our knowledge of equine respiratory lymphoid tissues.

Immunoglobulin producing plasma cells can be readily observed throughout the respiratory tract (RT) and specific antibody can be detected in mucosal secretions, for example after influenza infection (Hannant *et al.*, 1989). The relative proportion of IgA and IgG producing cells varies over the length of the RT. There is a predominance of IgA producing cells in the nares (roughly 75 %) which progressively decreases distally. The reverse is true for IgG producing cells with a predominance of IgG producing cells in the lower airways. This work was performed on older horses (aged 2-12 years), the situation may be different however in younger Thoroughbred horses where IgA may predominate along the entire RT (T. Blunden, personal communication).

Well defined lymphoid tissue has been shown throughout the RT of the horse. These lymphoid tissues are thought to be part of an adaptive CMIS and can be categorized into two histological features. Dense infiltrates of closely packed lymphocytes, plasma cells, and small numbers of neutrophils are observed as a cellular cuff around the epithelium and are distributed sparsely throughout the RT but extensively in the bronchae and bronchioles. Their numbers and size vary between individuals and are often seen in the interstitium of the lung commonly associated with blood vessels. Lymphoid follicles, very similar to GALT, with a modified epithelium are found in the nasal cavity, nasopharynx, around the opening of the auditory tube, in the trachea and occasionally in small bronchi (figure 1.3). The follicles are usually solitary in the nasal cavity, around the auditory tube, proximal trachea, and in the bronchi, whereas in the nasopharynx and larynx they often form multiple aggregated nodular lymphoid tissue.

As in other species, the lung epithelial cell of the horse constitutively expresses MHC class II (A.Hamblin, personal communication). In contrast with the surrounding areas, the epithelium overlying the dome area is thinner, non-ciliated and contains no goblet cells. The basement membrane is infiltrated by large numbers of small lymphocytes forming a

modified lympho-epithelium. The configuration of the epithelium in association with the follicles changes throughout the RT. For example, in the rostral areas of the nasopharynx, the epithelium shows a polypoid villus-like fold morphology, whereas in the nasopharyngeal tonsil, the nodules are more densely packed to form almost a monolayer of lymphoid tissue underlying the surface epithelium.

The dome areas underlying the lympho-epithelium contains small and large lymphocytes and a small number of plasma cells. Many nodules, especially in the nasopharynx show a large nodule of lymphocytes and macrophages which can be differentiated into a dark corona and a lighter-staining germinal centre containing mitotic small and medium lymphocytes and macrophages. The parafollicular area outside the nodules contains small lymphocytes, plasma cells, arterioles, high endothelial venules (HEV) and lymphatics.

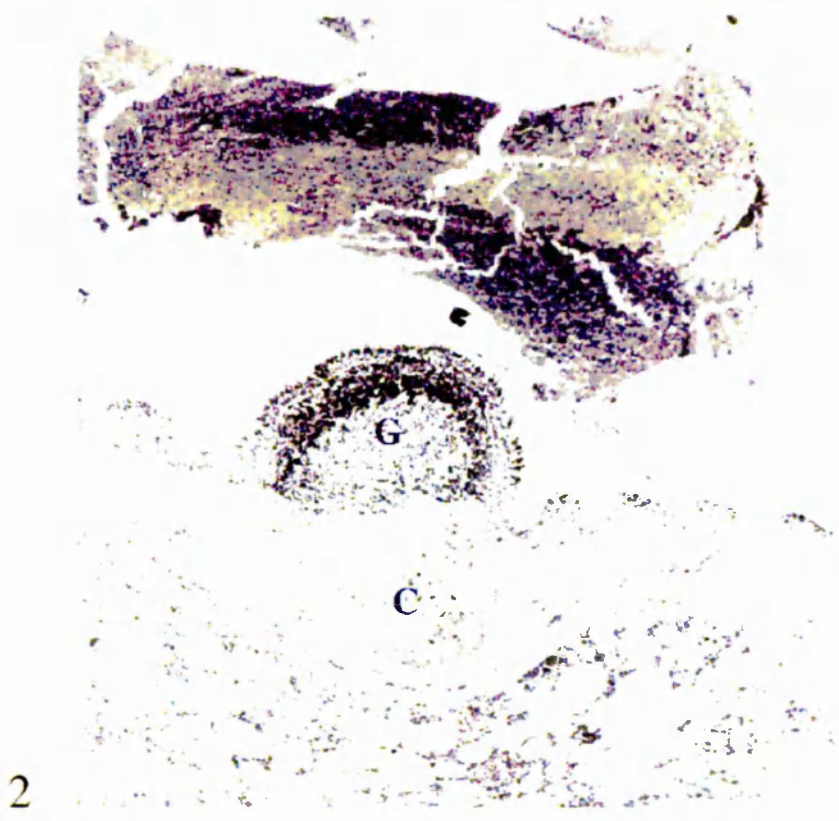
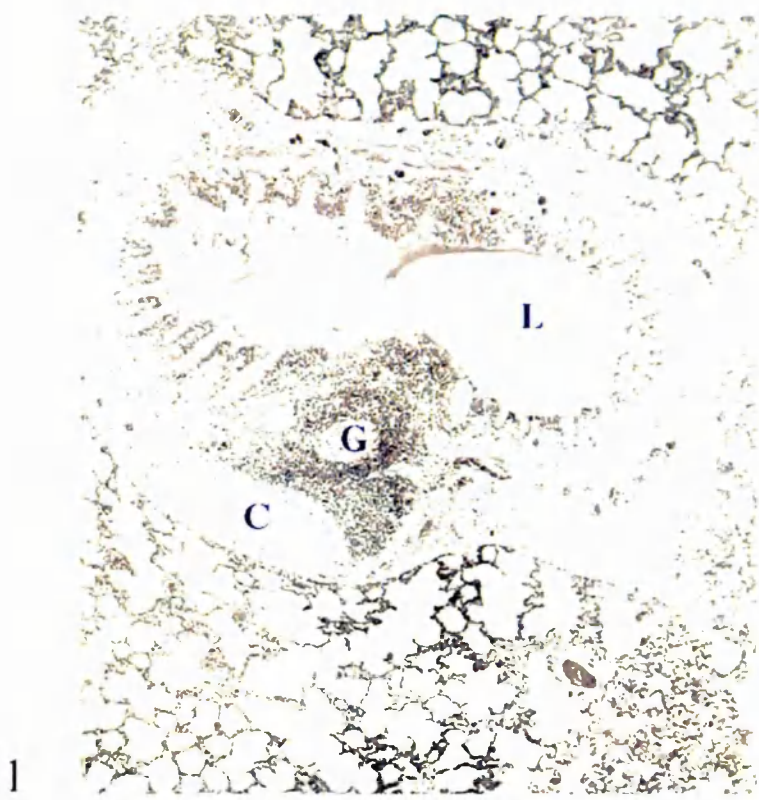
Broncho-alveolar lavage (BAL) fluids from the lung airways contain a large number of leucocytes even in the clinically healthy horse (Viel, 1983 and 1995). Approximately 60 % of these leucocytes are macrophages, and 36 % are lymphocytes (of which 95 % are T lymphocytes and 5 % are B lymphocytes). The ratio of CD4:CD8 T-cells in BAL fluids is less than or equal to 1 (Kydd *et al.*, 1996) in contrast to the peripheral blood where there is a larger proportion of CD4+ ve T cells and the CD4+:CD8+ ratio is approximately 3 (Lunn *et al.*, 1991; Kydd *et al.*, 1996). Small numbers of neutrophils (2.2%), mast cells (0.4%), and eosinophils (0.03%) are also found in normal equine BAL fluids (Viel, 1995; McGorum *et al.*, 1993; Kydd *et al.*, 1996).

During a respiratory infection the numbers of lung leucocytes may alter dramatically. BAL samples collected during the course of equine herpes virus 1 (EHV1) infection showed evidence of neutrophilia, lymphopaenia with a decline in macrophage numbers on day 2

Figure 1.3 Lymphoid follicles in the equine lung.

1. Bronchus with lymphoid tissue from a clinically normal 12 week old foal ($\times 140$).
2. Bronchus with lymphoid tissue from a clinically normal 3 year old colt ($\times 140$).

L, lumen; G, germinal centre, C, cartilage.



post infection. At days 21 and 28 there was a reduction of the CD4:CD8 ratio largely as a consequence of an increase in CD8 +ve T lymphocytes (Kydd *et al.*, 1996).

These data indicate that the equine RT is an adaptive immunological organ. The fact that there are selectively more CD8+ than CD4+ T cells within the lung compartment and the fact that they express IL-2 receptors and show lymphokine activated killer activity (LAK) (Kydd *et al.*, 1994), suggests that the lung is a site of dynamic immunological response. It possesses the histological apparatus of a common mucosal immune system similar to that found in humans and has been shown to respond to infections in terms of BAL cytology and local antibody responses. In humans, immunological responses at the level of the RT have been shown to be protective for many respiratory pathogens (Clements *et al.*, 1984 and 1986). The existence of activated immunological responses in the equine RT indicates a significant role of these mucosal immunological responses in the protection of the horse against pathogenic insult.

1.2 Aspects of mucosal vaccination

1.2.1 Introduction

The vast mucosal surface in mammals provides the site of infection for the majority of respiratory and gastrointestinal pathogens. It is now almost universally accepted that the mucosal immune system is best adapted to provide protection from mucosal pathogens thanks to the anatomical location of its effector mechanisms at the site of initial infection.

Since the 1970's there has been a concerted effort to investigate the common mucosal immune system (CMIS) and to develop effective methods of mucosal vaccination to combat mucosal pathogens (Ogra *et al.*, 1980). In general, the induction of sterile immunity against mucosal pathogens requires strong effector mechanisms at the level of the mucosal surface, these are most effectively achieved by administering immunogens by the mucosal route rather than parenterally (Mestecky, 1987).

1.2.2. Importance of mucosal effector responses

The effector arms of the CMIS include humoral (comprising mainly secretory IgA, sIgA), and cellular responses, including cytotoxic lymphocytes (CTL). There is a great deal of experimental evidence that IgA is the most important mediator of protection present at the mucosal surface. Passive administration of virus specific IgA into the nares of recipient mice is sufficient to protect them from experimental infection with a number of viruses, including sendai (Mazanec *et al.*, 1987) and influenza (Tamura *et al.*, 1990). Secretory IgA, but not monomeric IgG, when injected systemically into mice locates to the mucosal surface and protects the upper respiratory tract (URT) from influenza virus infection

(Renegar and Small, 1991). IgA may also be a major mediator of cross-protection from heterologous influenza infection (Liew *et al.*, 1984; Tamura *et al.*, 1990; Asanuma *et al.*, 1995). This situation has also been implied during human influenza vaccine trials where protection from infection is most strongly correlated with nasal HA specific IgA (Clements *et al.*, 1984 and 1986).

There have been many roles proposed for sIgA in the protection of the mucosal epithelium (Outlaw and Dimmock, 1990 and 1991). These include the creation of a mucosal barrier across which specific pathogens cannot pass and prevention of binding to the target cell (Lamm, 1976; Taylor and Dimmock, 1985; Mestecky and McGhee, 1987), intracellular neutralisation of viral growth during IgA transport (Mazanec *et al.*, 1995), and excretion of IgA-antigen complexes formed in the lamina propria after an infection (Kaetzel *et al.*, 1991, Lamm *et al.*, 1992).

1.2.3. Adjuvants and delivery systems

Unfortunately, simple application of non-replicating antigens onto the mucosal surface either orally or intranasally, rarely stimulates significant sIgA responses, and can lead to mucosal tolerance through the activation of CD8+ve suppressor T cells (Ke and Kapp, 1996). Thus, wide range of mucosal adjuvants and delivery systems have been developed to attempt to overcome these problems and increase the antigen specific immunological responses. The most important of these are discussed below.

Microencapsulation technologies

Microspheres

Some of the problems associated with oral vaccination can be circumvented by encapsulation of the antigen into microscopic capsules. The gastrointestinal tract is a very harsh environment, incorporating the antigen within a capsule protects it from enzymatic degradation and may selectively enhance the uptake of the antigen into the inductive sites of the CMIS.

Biodegradable microspheres are around 5-200 μm in diameter and made from synthetic polymers. Their use has been investigated by several groups for vaccination at mucosal surfaces (Maloy *et al.*, 1994; McQueen *et al.*, 1993; Moldoveanu *et al.*, 1993). The microspheres themselves can be manufactured easily (Aguado and Lambert, 1992; Morris *et al.*, 1994) and once dried are very stable allowing transport and delivery to the most remote regions of the world. Orally presented microspheres are selectively taken up into Peyer's patches by M cells (Eldridge *et al.*, 1990). The antigen is then slowly released ready for presentation to the immune system by diffusion through the matrix pores and by slow degradation of the microsphere itself (Morris *et al.*, 1994). The size of the microparticle plays an important role in its adjuvanticity. Smaller particles in the nanometre range are more efficient at inducing immunological responses than those in the micrometer scale, this may be due to an improved uptake of the smaller "nanospheres" by M cells (O'Hagan, personal communication).

By varying the proportions of the constituent polymers (polylactide and polyglycolide) the release period can be varied from a few days to up to one year (Aguado and Lambert, 1992). This opens up the possibility of using more than one preparation of microspheres

with different release rates in the same oral vaccine. This is the concept of controlled release vaccines where both priming and boosting doses of antigen can be given in the same application (Aguado, 1993).

Microspheres have been used successfully to elicit cytotoxic T cell and mucosal IgA responses against poorly immunogenic antigens such as ovalbumin (Maloy *et al.*, 1994), and when administered orally, they induced protective immune responses against viral (influenza, Modoveanu *et al.*, 1993) as well as bacterial (*E. Coli*, McQueen *et al.*, 1993) infections.

Liposomes

Liposomes have long been used as delivery systems for systemic vaccines, hormones, and genetic material. It was observed many years ago that phospholipids and other polar amphiphiles in an aqueous environment form closed spherical membranes comprising of a bimolecular sheet (lamellae) of lipids (Bangham *et al.*, 1995). Depending on the method of production liposomes can take various forms with sizes ranging from 20 nm to 200 μ m, comprising unilamellar or multilamellar sheets (see review by Gregoriadis, 1990).

The adjuvant action of liposomes in mucosal vaccination probably depends on the route of application, and whether the antigen is located within the lumen or associated with the membrane of the liposome (Shahum and Therien, 1994). Like microspheres, liposomes may protect an antigen through the harsh intestinal environment and be taken up by Peyer's patches (Childers *et al.*, 1990). Multilamellar liposomes create an antigen depot and slowly release their load as each layer is degraded (Aguado and Lambert, 1992). Antigens which are poorly immunogenic when administered by the oral route, e.g. soluble

ovalbumin (Vadolas *et al.*, 1995) and peptides (Friede *et al.*, 1994) can be rendered immunogenic by virtue of being bound to the membrane or entrapped within the lumen of the liposomes. Small liposomes (20nm-5µm) are efficiently taken up by antigen presenting phagocytic cells and may initiate T cell stimulation through the influence of IL-1 (Shahum and Therien, 1994).

A different method of adjuvant action has been described during immunisation of the total respiratory tract (RT, de Haan *et al.*, 1995a, 1995b). Instillation of liposomes into the RT was thought to abrogate the suppressive role of alveolar macrophages, such that simple application of inactivated influenza antigen was sufficient to induce an antigen specific immune response. This effect is independent of any positive effects on antigen presentation, as evidenced by the observation that instillation of liposomes up to 2 days prior to application of influenza antigen was sufficient to enhance the influenza specific immune response.

Liposomes represent a versatile carrier system which offers the possibility of supplementing the vaccine, and working additively, with other immunostimulators such as IL-2 (Abraham and Shah, 1992), monophosphoryl lipid A (Zhou and Huang, 1993), muramyl dipeptide (Nerome *et al.*, 1990), or cholera toxin B subunit (Zhou *et al.*, 1995). Mucosal vaccinations with liposomal antigen preparations have been used successfully to protect animals from bacterial (*Pseudomonas aeruginosa* Girod de Bentzmann *et al.*, 1994; *Bordatella pertussis* Guzman *et al.*, 1993; *Shigella flexneri*, Mallet *et al.*, 1995), and viral (measles: de Haan, 1995; influenza: El Guink *et al.*, 1989; Friede *et al.*, 1994; de Haan *et al.*, 1995) infections.

ISCOMs

Immune stimulating complexes (ISCOMs) are closely related to liposomes and are formed simply by the addition of the saponin Quil A to cholesterol, forming 40-90 nm cage-like structures (Morein *et al.*, 1984). ISCOMs have been used for mucosal vaccinations against numerous antigens (McI Mowat and Donachie, 1991) and can induce both IgA and IgG at the mucosal surface. The potency of ISCOMs to adjuvant immune responses after oral presentation can be attributed to their efficient uptake by M cells and macrophages as a consequence of their sub-micrometer size, and also the adjuvant effect of Quil A. A key feature of these vaccines is that after oral administration of non-replicating antigens, instead of the normal oral tolerance, the ISCOMs not only elicit increases in mucosal antibody but also induce systemic CTL responses (McI Mowat *et al.*, 1991; Lipford *et al.*, 1995).

In general, systemic vaccination does not induce antibodies at the mucosal surface. This is not always the case however, sine systemic vaccination of horses with inactivated equine influenza virus in the form of ISCOMs resulted in the stimulation of influenza specific nasopharyngeal antibody (Hannant *et al.*, 1988). The kinetics of antibody appearance and duration implied that a proportion of the antibody (30-60%) was locally produced.

Recombinant Vectors

Live attenuated vaccines and recombinant vectors expressing foreign genes are efficient at stimulating immunological responses at the mucosal surface after a topical application. This is probably because of their ability to mimic a natural infection and invade tissues,

producing large amounts of the expressed antigen, inducing endogenous processing and theoretically stimulating the immune system by through the MHC class I pathway.

Bacterial vectors

Carter and Collins (1974) showed that the initial site of *Salmonella typhimurium* infection was the Peyer's patch. As this is the major IgA induction site in the murine gut, this work sparked interest in using *Salmonella* mutants as mucosal vaccine delivery systems.

Several different types of genetically engineered live attenuated bacterial mutants have been developed which are effective as live vaccines, e.g. the PhoP mutant which is no longer able to survive in macrophages (Miller *et al.*, 1990), and the Δ aroA mutant of *S. typhimurium* which retains its ability to colonise Peyer's patches but is avirulent because of its requirement for *p*-aminobenzoic acid (PABA) which is not found in the animal host (Hoiseth and Stocker, 1981).

Recombinant *Salmonella* mutants can be constructed to express foreign antigens and have been used as mucosal vaccines via several topical routes (Hopkins *et al.*, 1995). Valentine *et al.* (1996) used an Δ aroA mutant transfected with the Simian Immunodeficiency Virus (SIV) p27 gag gene during a study of oral vaccination. The mutant was designed in such a way that expression of the SIV gene was up-regulated after being phagocytosed by a macrophage. In this way oral vaccination induced high levels of SIV specific mucosal sIgA and systemic CTL responses.

The *Salmonella* mutant vaccine delivery systems probably exert their adjuvant action by stimulating Th1 dominant responses. Van Cott *et al.* (1996) took T cells derived from PP after an oral vaccination with the attenuated recombinant *S. typhimurium* (BRD 847)

expressing a fragment of tetanus toxin (TT). The isolated T cells secreted increased quantities of INF- γ , IL-2, and IL-10 but not the classical mucosal cytokines IL-4 and IL-5. Increased levels of macrophage derived IL-6 was also reported. A single dose of this vaccine induced subclasses of immunoglobulins commonly associated with Th1 responses, i.e. TT specific serum IgG2a.

Viral vectors

Live viral vectors which mimic natural infections have also been investigated. The most widely used vector is vaccinia virus although others have proved successful. For example, Gallichan and Rosenthal (1995) constructed a recombinant adenovirus expressing Herpes Simplex Virus (HSV) gB protein to vaccinate mice intranasally, resulting in high titres of serum and vaginal HSV specific IgA and IgG.

Recombinant vaccinia viruses are attenuated in such a way as to allow replication and expression of the inserted gene. Sutter *et al.* (1994) developed a strain of vaccinia virus (MVA) which expressed all of its gene products but was not able to assemble into infectious virus. Intranasal instillation of this construct into mice, containing the genes for nucleoprotein (NP) and haemagglutinin (HA) of influenza virus, elicited serum HI antibody, CTL, and fully protected the animals from influenza infection.

As with liposomes and microcapsules, recombinant DNA technology offers the opportunity to deliver immuno-stimulating cytokines to the cells involved in the induction of the immune response. For example Janardhana *et al.* (1994) demonstrated that incorporation of the IL-5 gene into vaccinia virus increased the recruitment of antibody secreting cells to the lung.

Despite the promising results obtained with these types of vaccines, there remain major drawbacks in the use of live attenuated vectors. Any attenuated vector always carries a risk of reversion to its pathogenic wild-type. Furthermore, problems in isolating attenuated vectors from their wild-type parents creates problems of unpure attenuated vaccine strains. These dangers combined with those associated with the use of recombinant DNA in the generation of the genetically altered organisms, impart acknowledged safety risks on any future vaccine candidate. Such vaccines in today's world of ever tightening safety control face ever increasing problems in regulatory licensing of the product. Although, admittedly this problem may be less significant in the non-human field of vaccine development.

Another potential problem with live vectors is the fact that after an initial usage, the vector may elicit not only an immune response directed against its foreign gene, but also against itself. This may preclude the use of a particular vector for repeated vaccinations as long as there remains immunity to it. For example Rooney *et al.* (1988) showed that a previous experience of a vaccinia influenza vaccine decreased the HSV-specific immune responses elicited after a subsequent vaccinia HSV vaccination.

Recently, two very interesting departures from established vaccination procedures have broadened the range of vaccination regimes and delivery vehicles. Work in the expression of bacterial DNA in tobacco and potato plants has developed the possibility of using transgenic food to produce huge quantities of oral vaccine. By feeding mice with potato which expressed the *E. coli* heat labile toxin B chain (LTB) endotoxin gene, Haq *et al.* (1995) were able to elicit LTB-specific gut and serum neutralising antibodies.

Palatability, in these cases may hinder their use in humans as cooking may abrogate antigenicity. Studies are underway, however, in other foodstuffs such as the banana which

will circumvent these problems. This cheap method of producing large quantities of easily administered vaccine has a great potential use in developing countries.

Systemic vaccination with conventional vaccines does not usually stimulate significant antibody secretion onto the mucosal surface. Daynes (Daynes *et al.*, 1996) attempted to modulate the immune response after a systemic vaccination in order to stimulate mucosal antibody. T cells derived from lymph nodes associated with the mucosal surface express a different cytokine profile (Th2) compared with those of peripheral lymph node T cells (Th1). He found that after an intramuscular vaccination, the Th1 cytokine profile of the local lymph node could be switched to a Th2 profile if the vaccine was supplemented with the hormone 1 α ,25-dihydroxy vitamin D₃. Using this procedure, Hepatitis B specific IgA antibody was induced in the urogenital, lachrymal, faecal, and oral secretions of mice after vaccination in the hind footpad (Daynes *et al.*, 1996).

Nucleic Acid Vaccines

Intramuscular injection of naked DNA containing the nucleotide sequence encoding pathogen specific antigens results in uptake and expression of the protein by muscle cells (review Felgner and Rhodes, 1991) thereby eliciting humoral (Fynan *et al.*, 1993) and cytotoxic T cell responses (Ulmer *et al.*, 1993). Encapsulation of viral mRNA in liposomes also induces virus specific CTL after parenteral vaccination (Martinon *et al.*, 1993). These vaccination methods have been shown to confer protection from viral challenge (Webster *et al.*, 1994).

This field of vaccine research is newly emerging and does not benefit from the advantage of hindsight as seen in the field of live vaccines. As such, data on vaccine safety has to be

observed with the knowledge that only limited studies have been performed on a relatively small scale. It should be remembered there are potential safety hazards predicted with nucleic acid vaccines, such as inopportune vaccine incorporation into the host genome causing irreversible genetic damage. Such events could theoretically lead to cancer if the region responsible for a proto-oncogene is disrupted.

Mucosal delivery of DNA vaccines against viral antigens (Influenza HA) has also been shown to elicit mucosal and systemic antibodies that protect recipient mice from viral challenge (Fynan *et al.*, 1993).

This is an exciting new research field which may offer valuable possibilities to stimulate strong humoral and cell mediated responses to defined antigenic sequences. Potentially safe and stable polyvalent vaccines are also a very attractive prospect for the poorest parts of the world where multiple vaccinations are too costly. Single application of polyvalent recombinant DNA by nasal vaccination could theoretically protect an individual from many different mucosal infection

Cholera Toxin

Cholera toxin (CT) and its non-toxic subunit CTB are both well established, potent, mucosal adjuvants which exhibit a wide variety of stimulatory and inhibitory actions (for a review see Snider, 1995). They have been used by a number of groups in mucosal vaccines which are characterised by large mucosal sIgA and serum IgG1 responses and conferring protection from various viral (influenza, Tamura *et al.*, 1988; Bovine herpesvirus 1, Israel *et al.*, 1992) and bacterial pathogens (*Pseudomonas aeruginosa*, Abraham and Robinson, 1991; *Helicobacter felis*, Lee and Chen, 1994).

CT is a heterohexameric bacterial endotoxin normally produced in the intestinal lumen by *Vibrio cholerae* and shares 80 % homology with the heat-labile enterotoxin produced by *Escherichia coli* (LT). It consists of a toxigenic A chain (CTA) supported by a pentameric ring of B subunits (CTB). In the normal course of events the B pentamer binds GM1 ganglioside, present on the membrane of intestinal epithelial cells, and inserts the A chain into the cytosol of the target cell. Reduction of the A chain intracellularly releases an A1 fragment capable of binding NAD and catalysing the ADP ribosylation of Gs α , a GTP-binding regulatory protein associated with adenylate cyclase. The result is a sharp increase in cyclic AMP production (cAMP, Spangler, 1992).

The precise role of the two subunits in the adjuvanticity of CT is currently being evaluated. Each subunit CTA and CTB probably has individual mechanisms which can work independently or in cooperation with each other. For example, CTA increases intracellular cAMP, but this alone is not enough to induce an immune response. CTB mixed with the cAMP inducer forskolin similarly fails to stimulate an immune response. This observation suggests that mere binding of CTB to GM1 ganglioside or an increase in intracellular cAMP is not sufficient and a mixture of the two are required (Tamura *et al.*, 1995). It is clear nonetheless that some commercial sources of CTB can be used to optimal effect because of contamination with trace amounts (less than 0.1%) of CTA (Tamura *et al.*, 1994).

When used in mucosal vaccines CT can either be simply mixed or covalently conjugated with the antigen to exert its adjuvant effects. As mentioned previously, simple application of antigen to the mucosal surface may result in suppression of systemic responsiveness to that antigen, a mechanism termed "oral tolerance". CT and CTB have always been observed not to establish oral tolerance to themselves, and additionally have the effect

when co-administered with an unrelated antigen of “breaking” the establishment of oral tolerance to that antigen (Elson *et al.*, 1984). Recent findings have highlighted differences in the roles of the CT subunits and identified the need for care in this area. Intranasal vaccination with a CTB-antigen conjugate, in the absence of CTA, not only does not induce a mucosal immune response to the antigen but also enhances the antigen specific systemic tolerance (Sun *et al.*, 1994; Wu and Russell, 1994). It is suspected that the tolerance breaking properties, previously attributed to both CT and CTB, are selective for CT. Thus the conflicting literature based on commercially available CTB might be explained by low levels of contaminating CT (Czerkinsky and Holmgren, 1995).

CT has been demonstrated to exert wide-ranging effects at almost all stages of the immune response including uptake of antigen, stimulation of antigen presentation, T-helper stimulation and proliferation of B cells. It can also be used in the face of pre-existing anti-CT immunity (Tamura *et al.*, 1989; Wu and Russell, 1994). Once ingested, CT can be observed in the PP and intestinal LP within one hour (Lindner *et al.*, 1994). One of its first effects on the immunological responses to bystander antigens is that CT increases the permeability of the mucosal epithelium to luminal proteins (Lycke *et al.*, 1991; Gizurarson *et al.*, 1992).

Although CT has been shown to stimulate antigen presentation by boosting IL-1 production of APC's *in vitro* (Lycke *et al.*, 1989; Hirabayashi *et al.*, 1992) the major facets of its adjuvanticity probably lie with the regulation of the cytokine environment within the mucosal induction site and the inhibition of oral suppression. Cytokines commonly associated with a Th2 type profile (Mosmann and Coffman, 1989) have been shown to be important in the generation of IgA production (Murray *et al.*, 1987) and appropriately, PP have also been shown selectively to produce Th2 cytokines (Lagoo *et al.*, 1994; Hiroi *et al.*, 1995). In contrast with other vectors such as *S. typhimurium*, which specifically

enhance a Th1 type cytokine profile in the mouse gut (Klimpel *et al.*, 1995; Van Cott *et al.*, 1996), the cytokines stimulated by CT are of the Th2 type (IL-4 and IL-5: Xu-Amano *et al.*, 1994; IL-6 and IL-10: Klimpel *et al.*, 1995).

Once CT has reached the mucosal induction site it exerts regulatory effects on both T and B cells and succeeds in establishing immunological memory in both of these cell types (Vajdy and Lycke, 1992 and 1993; Jertborn *et al.*, 1994). Normal feeding of antigen inhibits priming of CD4+ve T cells and systemic antibody responses while activating CD8+ve T cells which may exhibit a suppressive function (Ke and Kapp, 1996). In contrast when CT is fed to mice it greatly promotes selective priming of CD4+ve T cells through the action of interleukins (IL)-2, 4, 5, 6, 10, and INF- γ (Hornquist and Lycke, 1993) and abrogates the induction of CD8+ve suppressor cells (Elson *et al.*, 1995). The responses of mucosal B-cells are also significantly enhanced. CT has been shown both *in vitro* and *in vivo* to induce isotype switching of IgM+ve B cells to produce IgA and IgG (Lebman *et al.*, 1988; Lycke and Strober, 1989; Chen and Strober, 1990). This effect is probably mediated by the actions of IL-4, IL-5, and IFN γ (Chen and Strober, 1989).

1.2.4. Summary

Mucosal application of antigen together with adjuvant has proved to be a powerful tool in the protection of animals from a wide variety of respiratory and gastrointestinal pathogens. Mucosal vaccinations have various advantages over their systemic counterparts. They may prevent the initial infection of the epithelium (Israel *et al.*, 1992; Takada and Kida, 1996), they may be responsible for greater cross protection from newly emergent virus strains (Liew *et al.*, 1984; Tamura *et al.*, 1990; Asanuma *et al.*, 1995), and

can be easily and rapidly administered by people that require no special training and are therefore ideally suited for use in developing countries (Aguado and Lambert, 1992).

Systemic vaccination, in some cases, may induce protective immunological responses in terms of protection from lethal challenge dose of pathogen. In a recent paper, Takada and Kida (1996) showed the importance of immunological mechanisms present at the mucosal surface. They observed that chickens immunised systemically were protected from challenge, yet still supported virus growth in the respiratory sinus. In contrast, intranasal vaccination of chickens induced mucosal immunological responses which prevented virus replication in sinuses.

The immunological mechanisms associated with the protection stimulated by a mucosal vaccination are located at the site of initial infection, and serve to prevent pathogen-cell contact or replication of infectious particles. This feature of mucosal immunity may be very important in the defence against pathogens that quickly cross the epithelial barrier and establish infections and latency elsewhere within the host. One example where this is the case is equine herpesvirus-1 (EHV-1). EHV-1 is observed to cause respiratory disease, cell associated viraemia in peripheral blood mononuclear cells, and establishes latency (Allen and Bryans, 1986). The virus crosses the epithelial barrier very rapidly such that antigen is detected in respiratory tract associated lymph nodes as soon as 12 hours after infection (Kydd *et al.*, 1994). The rapid intracellular localisation of EHV-1 indicates the important role of cellular immunity in early recognition of virus-infected cells and recovery from infection (Kydd *et al.*, 1994). However, work performed in cattle has shown that mucosal vaccination protects cattle from bovine herpesvirus-1 (BHV-1) infection, suggesting that a strong mucosal barrier present at the mucosal surface could prevent the penetration of virus and establishment of latency (Israel *et al.*, 1992).

1.3 Influenza

1.3.1 The influenza virus

Influenza virus is a member of the orthomyxoviridae family. The virion is a pleomorphic negative-stranded RNA virus of about 80-120 nm in diameter. The virus particle consists of a host cell derived lipid bilayer envelope encasing the viral genome associated core. The genomes of influenza viruses are comprised of eight single-stranded RNA chains together encoding at least ten different proteins (Skehel and Schild, 1971).

The outer lipid bilayer contains two glycoproteins, haemagglutinin (HA) and neuramidase (NA), which are embedded in the membrane via their hydrophobic ends (figure 1.4). Directly beneath this lipid envelope is a protein capsule made up of matrix (M) protein, which holds the RNA fragments connected to the nucleocapsid (NP) and polymerase proteins (P1, P2, and PA). The viral genome also encodes two non-structural proteins that are not incorporated into the virion (NS1 and M2).

Influenza viruses are classified on the basis of their type-specific internal M and NP proteins. Thus influenza B and C viruses are restricted to human infections, while influenza A viruses have been isolated from disease outbreaks in many animal species. Type A viruses are further subtyped depending on their specific glycoprotein content. Fourteen distinct HA and nine NA molecules have been identified in avian viruses, whilst only two types, H7N7 (A/equine/1) and H3N8 (A/equine/2) have been recognised in equine influenza outbreaks.

Haemagglutinin

Of the total viral protein HA makes up about 25%, it is the major membrane bound protein. It is found as 10-14 nm spikes distributed evenly over the surface of the virion. It is responsible for the attachment of the virus onto the target cell receptor, sialic acid, and also mediates fusion of the endocytosed virus with the endosomal membrane. As such HA is the major target for neutralising antibodies. The HA molecule is a trimer of identical non-covalently linked subunits (Wilson *et al.*, 1981). It is triangular in cross section for much of its length, but spreads out at its end to reveal the three constituent parts. Each subunit is synthesised as a single polypeptide chain but postranslational events cleave the N-terminal region leaving two proteins, HA1 (51 kDa) and HA2 (24kDa). Nucleotide sequences for the HA of both equine subtypes H3 and H7 have been determined (Daniels *et al.*, 1985; Gibson *et al.*, 1992; Binns *et al.*, 1993). While major antigenic differences have been identified between the subtypes, minor antigenic "drift" changes may occur within a subtype to allow the emergence of a new strains of influenza viruses (Daly *et al.*, 1996).

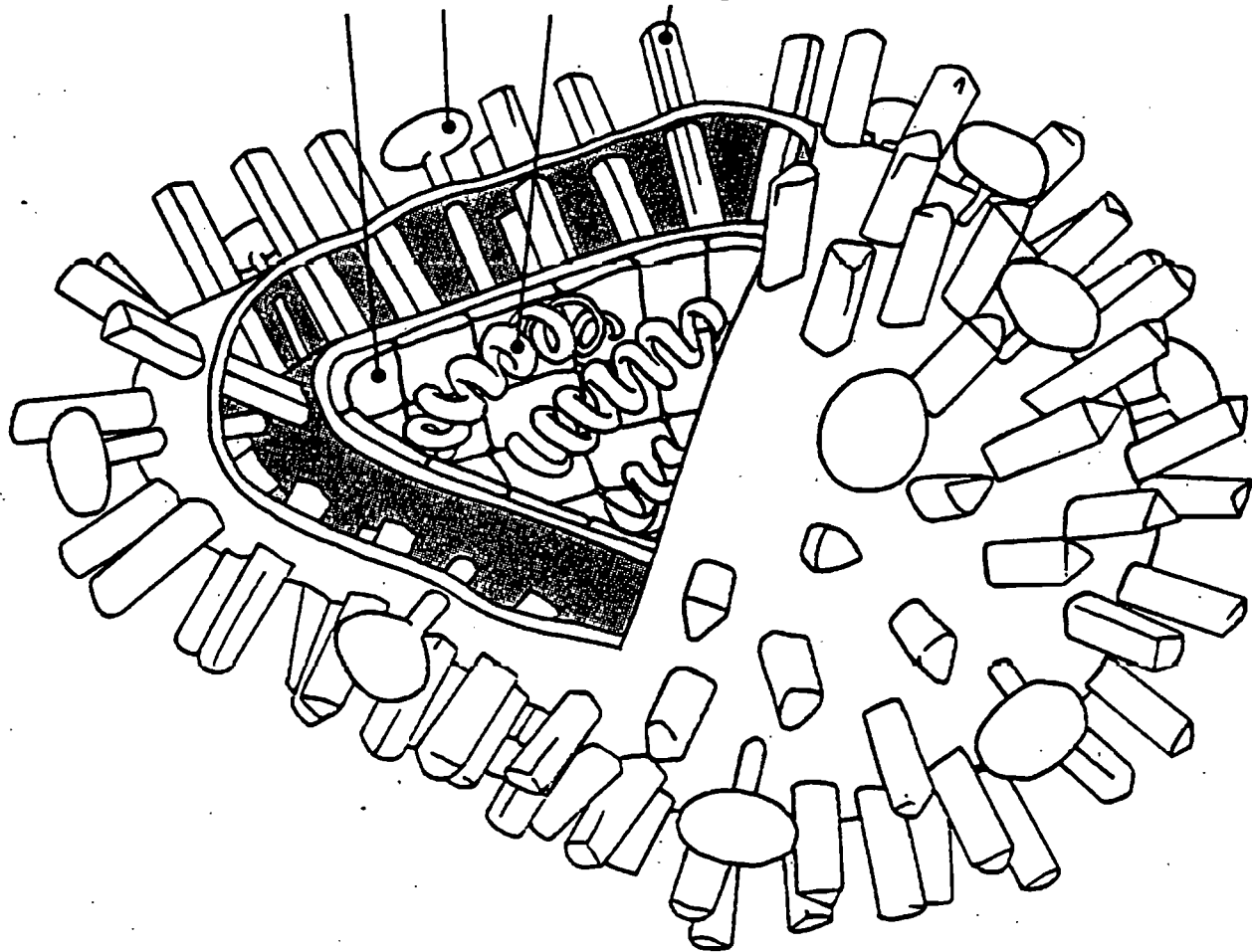
Neuramidase

Neuramidase (NA) is the second membrane bound glycoprotein on the viral surface, making up around 10 % of total viral protein. Its role is to cleave any terminal sialic acid residue from glycoconjugates (Gottschalk, 1957) and is therefore responsible for cleaving the HA of a newly formed virus from the sialic acid containing receptor on the host cell, thereby releasing the virus and preventing reabsorption into the cell (Griffin *et al.*, 1983).

Figure 1.4 Equine influenza virus.

A three dimensional diagram showing the structure of an influenza virion. M, M-protein; NA, neuramidase; RNP, ribo-nucleoprotein; HA, haemagglutinin.

M NA RNP HA



NA has also been implicated in the fusion of viral and cell membranes (White *et al.*, 1982). Also, NA may help the transmission of virus particles across the mucin secretion barrier present on respiratory epithelia by cleaving non-specific HA inhibitors (Klenk and Rott, 1988).

The mushroom shaped spikes of the NA molecule are made up of four identical subunits, each having a molecular weight of around 60 kDa. The NA molecule has a box-shaped head attached onto a thin stalk, the length of which appears to be related to host range and virus virulence (Castrucci and Kawaoka, 1993). Antigenically the NA is strain specific and displays antigenic variation within a subtype and major antigenic differences between the subtypes. Only two subtypes, N7 (A/equine/1) and N8 (A/equine/2), have been identified in equine influenza viruses.

Nucleoprotein

Nucleoprotein (NP) is a major group-specific internal protein that distinguishes between influenza A, B, and C viral types, whilst showing little variation between subtypes. It is a protein of around 60 kDa which associates, together with the polymerase molecules, and the viral RNA to form ribonucleoprotein (RNP) particles. As well as this structural role, NP is also involved in the replication of the viral genome (Skorko *et al.*, 1991). Diagnosis of equine influenza infection is routinely performed by detection of NP antigen by capture ELISA techniques from nasal swab samples (Cook *et al.*, 1988).

Matrix proteins

The matrix protein (M) is also a group-specific antigen, and is the most abundant internal protein associated with the influenza virus. This protein, of approximately 27 kDa, lines the inner surface of the lipid bilayer and accounts for up to 47% of the total virus protein. A second M protein (M2) is found in large quantities in the infected cell and small amounts in the intact virion. The M2 protein is a highly conserved transmembrane ion channel which serves to regulate the pH of the infected cell and resultant virion (Sugrue and Hay, 1991). Monoclonal antibodies against the M2 protein reduce viral growth in tissue culture (Zebedee and Lamb, 1988), and vaccination of mice with recombinant M2 protects them from heterologous influenza challenge (Slepushkin *et al.*, 1995).

The polymerase and non-structural proteins

Together with NP and viral RNA, three polymerase molecules P1, P2, and PA aggregate to form the RNP particles, and are the largest influenza virus proteins at 96, 87, and 85 kDa respectively. They are associated with the expression of the viral genome within the nucleus of the infected cell.

Two so-called non-structural proteins are found in large quantities in the infected cell NS1 and NS2 (23 and 11 kDa respectively). NS1 is only found in infected cells and is involved in the regulation of viral gene expression by controlling the export of mRNA from the cell nucleus. Qui and Krug (1994) postulated that accumulation of NS1 in the nucleus in the early stages of infection binding of the NS1 to the polyA tails would block the exit of all mRNA from the nucleus. The cellular mRNA would then be cleaved by the viral polymerases, leaving the capped primers free to be used for further viral mRNA synthesis.

The cleaved cellular RNA would be unable to leave the nucleus and would be degraded, in this manner the NS1 protein hijacks the cells protein synthesis apparatus for purely viral proteins.

NS2 protein is a protein formed from a spliced product of NS1 mRNA. It localises in large quantities in the nucleus of the infected cell and is thought to be involved in viral protein synthesis. It has also been detected in small amounts in purified viruses (Richardson and Akkina, 1990).

1.3.2 Equine influenza

Equine influenza was first recognised during a natural outbreak in 1956 (Sovinova *et al.*, 1956). This virus, designated A/equine/1/Prague/56 was identified as a H7N7 influenza virus. No H7N7 outbreak has been reported since 1979. In 1963, an influenza virus (A/equine/2/Miami/63, H3N8) was isolated which showed major antigenic differences from the Prague '56 virus (Scholtens and Steele, 1964) and since then, there have been sporadic epizootics of H3N8 equine influenza, with each responsible virus differing only slightly from the previous isolate due to antigenic drift (Daly *et al.*, 1996).

The virus is inhaled in microscopic aerosol and infects the ciliated epithelial cells of the upper respiratory tract (URT). Multiplication and viral release destroys these cells and the infection spreads throughout the URT including the nasal turbinates, nasopharynx, larynx, and trachea (Wilson, 1995). The function of the mucocilliary escalator becomes compromised due to deciliation of the epithelium from days 3-4 post infection (Sutton, personal communication, In Press 1997). The loss of mucus clearance allows invasion of opportunistic nasal bacteria, most commonly *Streptococcus zooepidemicus* or *Pasturella* /

Actinobacillus spp.. Inflammation of the lung leads to epithelial erosion and oedema of the mucosae. Bronchoalveolar lavage sampling over the course of an infection reveals a neutrophilia and depletion of macrophages between days 3-7 after infection, with no significant changes to lymphocyte numbers (Sutton *et al.*, 1996). Clinical signs might include, pyrexia (105 °C, days 3-7), dry non-productive cough, serous nasal discharge which may become purulent, lethargy, anorexia, increased breathing and heart rate, hyperaemia of the nasal and conjunctival mucosae, lacrimation, limb oedema, muscle soreness and spasms (Wilson, 1995).

1.3.3 Immunological responses to equine influenza infection

Experimentally induced influenza infection with reproducible clinical signs has been used to investigate the immunological responses to infection and to study the correlates of immunity (Mumford *et al.*, 1990). Primary infection with influenza results in stimulation of antigen specific IgG, IgA, and IgM antibodies in serum and nasopharyngeal and tracheal washes; although this antibody is rather short lived (Hannant *et al.*, 1988a and 1989a). Genetically restricted cytotoxic lymphocytes (CTL) have also been detected in the peripheral blood mononuclear cells (PBMC) 14 days after infection (Hannant *et al.*, 1988a and 1989b).

1.3.4 Immunological correlates of immunity to influenza infection

It has proved difficult to define the relative contribution of serum and mucosal antibodies and cellular immune responses to the protection of horses from influenza challenge. The functional significance of local antibody has been difficult to elucidate in the presence of

high levels of background serum antibody (Rouse and Ditchfield, 1970). In other species such as humans, influenza vaccine trials have indicated an important role for local antibody in protection from infection, and shown that protection is most strongly correlated with nasal HA specific IgA (Clements *et al.*, 1984 and 1986; Tomoda *et al.*, 1995). Passive administration of virus specific IgA into the nares of recipient mice before challenge protects them from experimental influenza infection (Tamura *et al.*, 1990). Also, influenza specific secretory (s)IgA injected into mice can be observed to locate to the mucosal surface and protect the URT from infection (Renegar and Small, 1991). sIgA may also be a major mediator of cross-reactive protection from heterologous influenza infection (Liew *et al.*, 1984; Tamura *et al.*, 1990; Asanuma *et al.*, 1995). There have been many roles proposed for sIgA in protection of the mucosal epithelium against influenza infection (Outlaw and Dimmock, 1990 and 1991). These include the creation of a mucosal barrier across which virions cannot pass and prevention of binding to the target cell (Taylor and Dimmock, 1985), intracellular neutralisation of viral growth during IgA transport (Mazanec *et al.*, 1995), and excretion of IgA-antigen complexes formed in the lamina propria after an infection (Kaetzel *et al.*, 1991).

After an influenza infection, equids exhibit clinical immunity to rechallenge with homotypic virus for up to 1 year in the absence of residual circulating antibody, suggesting a role for other mechanisms of immunity (Hannant *et al.*, 1988b). This feature contrasts with the situation after vaccination with inactivated antigens, where there is a strong correlation between protection from homologous virus infection and the amount of circulating antibody directed against the virus HA (Mumford *et al.* 1983, 1988). However, once this serum antibody has declined, the animals become susceptible to infection.

Equine influenza-specific CTL has been observed after infection (Hannant *et al.*, 1988a and 1989b), but their role in protection from rechallenge has not been studied in detail.

Influenza-specific CTL in other species have been extensively reviewed (Askonas *et al.*, 1982; McMichael, 1994). Briefly, CTL responses have been detected against the majority of structural and non-structural influenza proteins including, HA (Zweerink *et al.*, 1977), NP (Yewdell *et al.*, 1985), M1 (Gotch *et al.*, 1987) and the polymerase molecules and NS1 (Bennink *et al.*, 1987). CTL responses against conserved proteins such as NP and M protein are thought to be the main mediators of cross-reactive cytotoxicity. HA-specific CTL probably only contribute to the immunological effector response against homologous virus (Zweerink *et al.*, 1977). While there are reports of CTL alone conferring protection from influenza infection (McMichael *et al.*, 1983a), they are commonly thought to be responsible for virus clearance during the recovery phase of infection, and thereby limiting clinical disease (McMichael, 1994).

As neutralising antibody is directed primarily against HA, antigenic and genetic variation of influenza viruses over time renders the protection engendered by humoral responses, induced by infection or vaccination, progressively less efficient (McMichael, 1994). However, cross-reactive CTL still recognise these emergent viruses due to their restriction for conserved proteins such as M and NP. In humans the immunological memory of these CTL can be as long as 4 years (McMichael *et al.*, 1983b). This persistent quasi-immunity of inefficient humoral activity and long cytotoxic T cell memory gives rise to problems in heavily vaccinated populations such as the equidae. Vaccination with vaccines containing antigenically distant viral strains induces humoral immune responses which may be inefficient in neutralising newly emergent viral strains, thereby potentially allowing infections of vaccinated horses which are sub-clinical and permit nasal virus shedding to further propagate the virus outbreak (Mumford, 1992).

1.4 Perspectives for mucosal vaccination

The important role of mucosal immunity in protection from human influenza (Clements *et al.*, 1984 and 1986; Tomoda *et al.*, 1995) has spurred interest in mucosal vaccination with inactivated influenza antigen and the potent mucosal adjuvant CT (Tamura *et al.*, 1988, 1990, 1994, 1995; Hirabayashi *et al.*, 1992). From close examination of the literature, it has become clear that mucosal vaccinations comprising CTB and inactivated influenza antigens could hold several advantages over conventional vaccines in horses.

1) With conventional vaccines, which rarely induce any antibody at the mucosal surface, an infection can only be arrested once the virus has infected and established itself within an epithelial cell. Systemic vaccination with an antigenically distant virus strain may allow a sub-clinical heterologous infection to develop, during which virus may be shed from the nares of infected individuals. In this manner, an influenza outbreak may be propagated within populations of heavily-vaccinated animals (Mumford, 1992). A nasopharyngeal influenza vaccine could induce large quantities of type specific and cross-reactive IgA at the mucosal surface. This would prevent viral growth (Outlaw and Dimmock, 1990 and 1991; Taylor and Dimmock, 1985; Mazanec *et al.*, 1995; Kaetzel *et al.*, 1991), and also "mop up" free virus, thereby preventing nasal shedding.

2) It has been shown by many groups that protection from influenza infection with a heterologous virus is due, to a large extent, to sIgA present at the mucosal surface (Liew *et al.*, 1984; Tamura *et al.*, 1990; Renegar and Small, 1991; Asanuma *et al.*, 1995). Generation of influenza-specific sIgA is therefore a desirable goal of any successful influenza vaccine. This can best be achieved by vaccination via the mucosal surface (Mestecky, 1987). This function of sIgA to prevent infection with heterologous virus may

be accounted for by the hypothesis that some of the mechanisms by which sIgA may prevent virus infection depend on its physical attachment to the virion and may be independent of the role of the target antigenic domain/epitope. The mechanism for this is purely speculative, potential mechanisms include locking virus in association with the mucus layer thus preventing attachment to epithelial cells (Israel *et al.*, 1992), binding to viral particles whilst still inside the epithelial cell and transporting the antibody/antigen complex across the epithelium and releasing it into the airway lumen (Mazanec *et al.*, 1995); and the neutralisation of free viral particles in the lamina propria and subsequent excretion through the secretory component translocation mechanism (Kaetzel *et al.*, 1991). These mechanisms may depend on mere binding of IgA to the pathogen and not blocking any one particular function of the pathogen.

Thus establishment of IgA responses capable of binding newly emergent viral strains, as described above, may have a role in preventing the sub-clinical infections which otherwise may be permitted in the presence of immunoglobulins which neutralise virus via the blocking of a specific target epitope.

3) During an equine influenza infection, the recall of anamnestic immunological responses at the level of the mucosal surface is extremely rapid. The appearance of influenza specific immunoglobulins in the nasopharynx of these horses occurs within 4 days of the infection (Hannant *et al.*, 1989a). It is thought therefore, that a primed equine mucosal immune system has the potential to mount a protective immunological response very quickly after antigenic presentation. This feature of the mucosal immune system would allow a mass vaccination programme of stable yards, to induce swift protective and cross-reactive immunoglobulin responses, in the face of an epizootic.

1.5 Objectives of the study

The aims of this study are three fold:

- (i) To investigate the existence of the common mucosal immune system in the horse by observation of immunological responses after intranasal vaccination.
- (ii) To induce influenza specific antibody responses at the mucosal surface and demonstrate anti-viral activity of those antibodies.
- (iii) To investigate the efficacy of intranasally administered inactivated influenza antigen together with cholera toxin B chain, as a vaccination regime to protect horses from influenza infection.

Chapter 2

Development and validation of methods to
measure antibody levels and function in nasal
washes

Chapter 2. Development and validation of methods to measure antibody levels and function in nasal washes.

2.1 Introduction

The investigation of the mucosal immune system in a large animal like the horse, and its immunological responses after infection and vaccination, is furnished with problems of reproducible sampling. Generally, mucosal secretions have been collected by extraction from absorbent swabs which were left in the nasal passage of the horse for long periods of time (Rouse and Angulo, 1970). This technique causes discomfort to the animal, and could cause erosion of the epithelium leading to "leakage" of systemically derived material into the nasal swab.

We have used a nasopharyngeal lavage technique to obtain antibody specifically from the nasopharynx and nasal passages. However, samples collected in this way may inherently exhibit great variation in the concentration of components in the resultant nasal wash because of sampling manipulation error, uncooperative experimental animal, or other effects on the rate of production of nasal secretions (e.g. intercurrent respiratory disease). To overcome these effects, and allow interpretation of the resultant ELISA data, methods of standardising each nasal wash have been developed.

Many studies have been published on intranasal vaccination against influenza in other species which showed vaccination-induced antigen-specific mucosal immune responses. However, only rarely have these responses been demonstrated to possess anti-viral activity (Tamura *et al.*, 1990). A major aim of this project was therefore to develop an assay to measure influenza virus-specific neutralising antibody in nasopharyngeal washes.

2.2 Materials and methods

2.2.1 *Collection of nasal wash samples*

Ponies that were to have their nasopharynx lavaged were sedated using 0.2 ml of intravenous Domosedan [*Veterinary Drug company*]. After sedation was achieved a short length (roughly 6 inches) of clear PVC tubing (Portex, 6.5mm external diameter \times 2.5 mm internal diameter) was inserted into one nostril of the horse and 50 ml of sterile PBS was instilled with a 50 ml syringe. The nasal wash lavage fluid was caught in a large sterile beaker as it dripped from the nostrils.

The method of nasal washing evolved over the course of these studies. An improved apparatus was developed for both nasal lavage and vaccine intillation (as described in chapter 4). The preferred apparatus is constructed with a spray nozzle at one end and is inserted to a point deemed to be at the entrance to the opening of the nasopharynx (see 4.2.1). A good placement of the tubing was shown by a continuous stream of lavage fluid exiting the nasopharynx from both nostrils. Once back in the laboratory, the nasal washes were vortexed and clarified by centrifugation (1500 \times g, 5 mins). The supernatants were then collected and frozen at -20°C.

2.2.2 *Collection of Bronchoalveolar lavage (BAL) samples*

The bronchoalveolar spaces of ponies were sampled using a variation of a blind lavage method first described by Dyer *et al.*, (1983) and used in this department (Kydd *et al.*, 1996).

2.2.3 ELISA to determine the influenza / cholera toxin specific antibody in nasal washes / serum

A 96 well ELISA plate (Immulon 3, [Dynatech, cat. no. 011-010-3755]) was coated overnight, at 4°C with antigen diluted to 20 µg/ml in 0.05M carbonate/bicarbonate buffer pH 9.6 [Sigma, cat. no. C-3041]. Antigen was either cholera toxin B chain or influenza antigen (as described in 5.1.2.1). The plate was incubated for 1 hour at 37°C, in blocking buffer (phosphate buffered saline [Unipath Ltd, cat.no. BR14]), containing 0.05% Tween 20 [Sigma, cat.no. P-1379], and 2% dried skimmed milk [Biorad, cat.no. 170-6404]) to block all free sites. The plates were washed thrice with washing buffer (phosphate buffered saline [Unipath Ltd, cat.no. BR14], containing 0.05% Tween 20.

Nasal wash samples were added in duplicate and titrated from undiluted sample by double dilution to 1/128 across the plate. Sera were added at 1/50 dilution initially and similarly titrated. The plates were incubated for 1 hour at 37°C and then washed thrice in washing buffer. To determine the antigen specific IgA, a monoclonal antibody (K129-3E7, kindly supplied by C.R. Stokes) which had been biotinylated using a commercial biotinylation kit [Amersham, cat. no. RPN 2202] was added diluted 1/300 in blocking buffer. To determine the antigen specific IgG, an affinity purified biotinylated goat anti-equine IgG (γ) [Dynatech, K&P, cat.no. 16-21-02] was used, diluted 1/1000 in blocking buffer. The plates were incubated for 1 hour at 37°C and then washed thrice in washing buffer. Streptavidin-horseradish peroxidase, (1:1000 [Amersham, cat.no. RPN 1231]) diluted in blocking buffer, was added to all wells and incubated for 1 hour at 37°C. The plates were washed thrice with washing buffer and the chromagen O-phenyldiamine (OPD [Sigma, cat.no. P6912]) diluted to 0.5 mg/ml in phosphate-citrate buffer, with sodium perborate [Sigma, cat.no. P-4922], was added. After 20 minutes for the colour to develop the

reaction was stopped, with 4M H₂SO₄, and the absorbence read at 490 nm (Dynatech ELISA plate reader, DIAS system).

2.2.4 ELISA to determine total IgA in a nasal wash sample

A 96 well ELISA plate (Immulon 4, [Dynatech, cat. no. 011-010-3855]) was coated overnight, at 4°C, with a monoclonal anti-equine IgA antibody (K129-2G5, kindly supplied by C.R. Stokes) diluted in 0.05M carbonate/bicarbonate buffer (pH 9.6 [Sigma, cat. no. C-3041]). The plate was incubated for 1 hour, at 37°C, in blocking buffer ((phosphate buffered saline [Unipath Ltd, cat.no. BR14], containing 0.05% Tween 20 [Sigma, cat.no. P-1379], and 2% dried skimmed milk [Biorad, cat.no. 170-6404]) to block all free sites. The plates were washed thrice with washing buffer (phosphate buffered saline [Unipath Ltd, cat.no. BR14], containing 0.05% Tween 20 [Sigma, cat.no. P-1379]).

Nasal wash samples were added and titrated across the plate from neat to 1/64. The plates were incubated for 1 hour at 37°C and then washed thrice in washing buffer. A second monoclonal antibody (K129-3E7, kindly supplied by C.R. Stokes) which had been biotinylated using a biotinylation kit [Amersham, cat. no. RPN 2202] was added diluted 1/300 in blocking buffer. The plates were incubated for 1 hour at 37°C and then washed thrice in washing buffer. Streptavidin-horseradish peroxidase, (1:1000 [Amersham, cat.no. RPN 1231]) diluted in blocking buffer, was added and incubated for 1 hour at 37°C. The plates were washed thrice with washing buffer and the chromagen O-phenyldiamine (OPD [Sigma, cat.no. P6912]) diluted to 0.5 mg/ml in phosphate-citrate buffer, with sodium perborate [Sigma, cat.no. P-4922], was added. After 20 minutes for the colour to develop the reaction was stopped, with 4M H₂SO₄, and the absorbence read at 490 nm (Dynatech ELISA plate reader, DIAS system).

2.2.5 ELISA to determine total IgG in a nasal wash sample

A 96 well ELISA plate (Immulon 3, [Dynatech, cat. no. 011-010-3755]) was coated overnight, at 4°C, with a polyclonal rabbit anti-equine IgG (whole molecule) antibody [Sigma, cat. no. H9015] diluted in 0.05M carbonate/bicarbonate buffer (pH 9.6 [Sigma, cat. no. C-3041]). The plate was incubated for 1 hour, at 37°C, in blocking buffer (phosphate buffered saline [Unipath Ltd, cat.no. BR14], containing 0.05% Tween 20 [Sigma, cat.no. P-1379], and 2% dried skimmed milk [Biorad, cat.no. 170-6404]) to block all free sites. The plates were washed thrice with washing buffer (phosphate buffered saline [Unipath Ltd, cat.no. BR14], containing 0.05% Tween 20 [Sigma, cat.no. P-1379]).

Nasal wash samples were added and titrated across the plate from undiluted to 1/64. The plates were incubated for 1 hour at 37°C and then washed thrice in washing buffer. An affinity purified biotinylated goat anti-equine IgG (γ) [Dynatech, K&P, cat.no. 16-21-02] was then added to all wells, diluted 1/1000 in blocking buffer. The plates were incubated for 1 hour at 37°C and then washed thrice in washing buffer. Streptavidin-horseradish peroxidase, (1:1000 [Amersham, cat.no. RPN 1231]) diluted in blocking buffer, was added and incubated for 1 hour at 37°C. The plates were washed thrice with washing buffer and the chromagen O-phenyldiamine (OPD [Sigma, cat.no. P6912]) diluted to 0.5 mg/ml in phosphate-citrate buffer, with sodium perborate [Sigma, cat.no. P-4922], was added. After 20 minutes for the colour to develop the reaction was stopped, with 4M H₂SO₄, and the absorbence read at 490 nm (Dynatech ELISA plate reader, DIAS system).

2.2.6 ELISA to determine total immunoglobulin in a nasal wash sample

A 96 well ELISA plate (Immulon 3, [Dynatech, cat. no. 011-010-3755]) was coated overnight, at 4°C, with a polyclonal rabbit anti-equine IgG (whole molecule) antibody [Sigma, cat. no. H9015] diluted in 0.05M carbonate/bicarbonate buffer (pH 9.6 [Sigma, cat. no. C-3041]). This antibody was known to cross react with equine IgA. The plate was incubated for 1 hour, at 37°C, in blocking buffer (phosphate buffered saline [Unipath Ltd, cat.no. BR14], containing 0.05% Tween 20 [Sigma, cat.no. P-1379], and 2% dried skimmed milk [Biorad, cat.no. 170-6404]) to block all free sites. The plates were washed thrice with washing buffer (phosphate buffered saline [Unipath Ltd, cat.no. BR14], containing 0.05% Tween 20 [Sigma, cat.no. P-1379]).

Nasal wash samples were added and titrated across the plate from undiluted to 1/64. The plates were incubated for 1 hour at 37°C and then washed thrice in washing buffer. A polyclonal rabbit anti-equine IgG (whole molecule) horseradish peroxidase conjugate [Sigma, cat. no. A-9292] was then added to all wells and incubated for 1 hour at 37°C, diluted 1/1000 in blocking buffer. The plates were washed thrice with washing buffer and the chromagen O-phenyldiamine (OPD [Sigma, cat. no. P6912]) diluted to 0.5 mg/ml in phosphate-citrate buffer, with sodium perborate [Sigma, cat. no. P-4922], was added. After 20 minutes for the colour to develop the reaction was stopped, with 4M H₂SO₄, and the absorbance read at 490 nm (Dynatech ELISA plate reader, DIAS system).

2.2.7 Competition ELISA to determine equine serum albumin (ESA) levels in nasal washes

A 96 well ELISA plate (Immulon 3, [Dynatech, cat. no. 011-010-3755]) was coated overnight, at 4°C, with equine serum albumin (ESA, [Sigma, cat. no. A3434]) diluted to

20 µg/ml in 0.05M carbonate/bicarbonate buffer (pH 9.6 [*Sigma, cat. no. C-3041*]). The plate was incubated for 1 hour, at 37°C, in blocking buffer ((phosphate buffered saline [*Unipath Ltd, cat.no. BR14*], containing 0.05% Tween 20 [*Sigma, cat.no. P-1379*], and 2% dried skimmed milk [*Biorad, cat.no. 170-6404*]) to block all free sites. The plates were washed thrice with washing buffer (phosphate buffered saline [*Unipath Ltd, cat.no. BR14*], containing 0.05% Tween 20 [*Sigma, cat.no. P-1379*]).

In a separate 96 well plate, nasal wash samples were added in triplicate and a titration of ESA was set up in 65µl volumes to act as a standard curve. 65 µl of a 1/500 dilution of a polyclonal rabbit antiserum, prepared within the department and shown to be specific for ESA (Hannant *et al.*, 1989a), was added to all wells. After an incubation of 1 hour at 37°C, 100µl volumes were transferred from this plate to the corresponding well of the ESA coated plate. The plate was incubated for 1 hour at 37°C and washed thrice with washing buffer. A polyclonal goat anti-rabbit IgG (whole molecule) horseradish peroxidase conjugate [*Sigma, cat. no. A8275*] was diluted 1/1000 in blocking buffer and added to all wells. After an incubation period of 1 hour at 37°C, the plates were washed thrice with washing buffer and the chromagen O-phenyldiamine (OPD [*Sigma, cat.no. P6912*]) diluted to 0.5 mg/ml in phosphate-citrate buffer, with sodium perborate [*Sigma, cat.no. P-4922*], was added. After 20 minutes for the colour to develop the reaction was stopped, with 4M H₂SO₄, and the absorbence read at 490 nm (Dynatech ELISA plate reader, DIAS system).

An analysis of the results from the standard curve was performed using a 4-parameter logistic model. The mean ELISA data of the nasal wash samples (n=3) were calculated and using this figure the concentration of ESA present in the nasal wash was determined from the standard curve.

2.2.8 *Preparation of influenza antigen used in the HI assay*

Allantoic fluid containing A/Equi/2/Sussex/89 was grown as described in 5.1.2.1. To 40 ml of infectious allantoic fluid, 500µl of a 10% v/v suspension of Tween 80 [*BDH AnalaR*] in PBS was added. After 5 minutes, 20ml of diethyl ether [*BDH AnalaR*] was added and the mixture stirred for 15 minutes at 4°C. The layers were allowed to separate and the virus containing aqueous layer removed and stored to allow any excess ether to evaporate overnight. The antigen prepared in this way was aliquoted into soda glass vials and stored at -70°C.

Prior to use in the HI assay, the amount of haemagglutination (HA) units in the virus preparation was determined. In a 96 well V-bottomed plate, 25 µl of antigen was titrated across the plate in doubling dilutions. The final volume was made up to 50µl by the addition of 25µl of PBS. 50µl of a 1% suspension of chicken red blood cells (RBC) was added and after an incubation of 30 minutes at room temperature the plate was tilted and the HA concentration was read. The last dilution where agglutination was complete was assigned 1 HA unit (HAU).

2.2.9 *Haemagglutination inhibition (HI) assay*

Non-specific inhibitors of HA in serum were inactivated with heat treatment in the presence of potassium periodate. Serum samples (150µl) were incubated with 300µl of freshly prepared potassium periodate (0.016M [*BDH AnalaR*]) for 15 minutes at room temperature. 150µl of 3% v/v glycerol [*BDH AnalaR*] in PBS was added and incubated for a further 15 minutes. The serum samples were then incubated at 56°C for 30 minutes.

Nasal wash samples were dialysed against 2000 volumes of PBS for 2 hours at 4°C, and passed through a 0.22µm filter.

The inactivated sera, and sterile nasal wash samples, were titrated by doubling dilutions in PBS in V-bottomed microtitre plates in a volume of 25µl. To each dilution was added 25µl of Tween 80/ether-treated influenza virus (A/Equi/2/Sussex/89) diluted to 4 HAU (see 2.2.8). After 30 minutes at room temperature, 50µl of a 1% suspension of RBC were added to each well. The plates were then incubated for 30 minutes at room temperature. The HI titre of each serum/nasal wash was read to be the reciprocal of the highest dilution causing inhibition of the haemagglutination reaction (indicated by the ability of the RBC to run to the bottom of the well when the plate was tilted to an angle of 70°).

2.2.10 *Influenza virus neutralisation assay in tissue culture*

In a 96 well plate, oriented so that it had 8 columns and 12 rows, a half log titration of virus was set up beginning in row 1 (1:50 dilution). The virus used was A/Equi/2/Sussex/89, first isolated and passaged thrice in fertile hens eggs, which had subsequently been tissue-culture adapted by 10 low dilution passages in Madin Derby Canine Kidney (MDCK) cells [*Imperial Laboratories, NBL-2(MDCK)*].

Into this virus dilution series an equal volume of either serum free medium 199 ([*Sigma, cat.no. M-0148*], containing 125 µg/ml of trypsin [*Sigma, cat.no. T-8918*], 2 mM/ml L-glutamine [*ICN Flow, cat. no.10-801-49*], 100 IU/ml penicillin [*ICN Flow cat. no.16-702-49*], 100 µg/ml streptomycin [*ICN Flow, cat. no.16-702-49*] and 1% v/v of 7.5% sodium bicarbonate solution [*ICN Flow, cat. no.16-883-49*]), or a nasal wash sample was added to

all the wells of one set (in replicates of 4). The plates are then incubated for 1 hour at 37°C. Nasal wash samples were concentrated $\times 3$ using aquacide II [Calbiochem, cat. no. 17851] and sterilised through a 0.22 μ m filter prior to use.

MDCK cells were grown until confluent in 96 well flat bottomed tissue culture plates containing minimal essential medium (MEM [ICN Flow, cat. no. 12-102-54] containing 10% foetal calf serum [Sigma cat. no. F-2442], 2 mM/ml L-glutamine [ICN Flow, cat. no. 10-801-49], 100 IU/ml penicillin [ICN Flow cat. no. 16-702-49], 100 μ g/ml streptomycin [ICN Flow, cat. no. 16-702-49], 1% v/v $\times 100$ non-essential amino acids [ICN flow, cat. no. 16-810-49] and 1% v/v of 7.5% sodium bicarbonate solution [ICN Flow, cat. no. 16-883-49]). The plates were then washed twice in sterile PBS. Using a multi-channel pipette, 100 μ l of the virus dilutions were transferred from the dilution plate into the corresponding wells containing MDCK cells and the plates were incubated for 1 hour at 37°C. The plates were then aspirated and washed twice with sterile PBS. 100 μ l of serum free M199 was then added to all wells and the plates incubated for 72 hours in 5% CO₂, at 37°C.

After the incubation period, the plates are centrifuged gently (50 \times g, 2 mins), and virus was detected by its haemagglutination (HA) activity, performed on aliquots of supernatant aspirated from each well. The TCID₅₀ per ml of the virus, used as a reference control, was read from the set of virus titration containing virus and M199 only. The result was calculated using the Karber formula (Dougherty, 1964). The sets of virus titrations containing influenza neutralising antibody will give an apparent TCID₅₀ which is less than that of the reference control. The apparent TCID₅₀ of the antibody neutralised virus is subtracted from the TCID₅₀ of the reference control to give a neutralisation index (NI).

2.2.11 Protein assay

The protein concentration of nasal wash samples was determined using a commercially available kit [*Bio-Rad, cat. no. 500-0006*], by comparison with a standard curve of ESA. This method is based on the Bradford protein assay and was performed according to manufacturer's recommendations.

2.3 Results

2.3.1 Nasal wash immunoglobulins measured by ELISA

Nasal washes were taken during an intranasal vaccination study (described in 5.2). In this study three ponies (A58, D70, and 26E) received three doses of an intranasal CTB/influenza based vaccine before going onto an experimental infection. The results displayed in figures 2.1 and 2.2 indicate day 0 to be the day of the third application of vaccine, this day was 35 and 31 weeks after the first and second application of vaccine respectively. The animals were challenged using a technique described in Mumford *et al.*, 1990; briefly, 20ml of a dilution of influenza virus containing hens egg allantoic fluid was nebulised into a sealed room such that the ponies were exposed to 10^4 times a predetermined minimal infectious dose of influenza virus. A control pony, 001, received CTB alone on the same days as the vaccinates. Ponies P16 and P18 were the negative controls and received nothing at all.

Influenza specific antibody (IgA and IgG) present in the nasal washes was determined by ELISA (for method see 2.2.3), and expressed as a function of the mean of the group day 0

Figure 2.1 Raw influenza specific IgA and IgG in nasal washes after vaccination and infection.

Figure 2.1 follows the increases in influenza specific IgA (graph A) and IgG (graph B) in nasal washes after intranasal vaccination (day 0, open arrow) and experimental infection (day 22, closed arrow).

■ 001 ○ D70 ● 26E □ A58

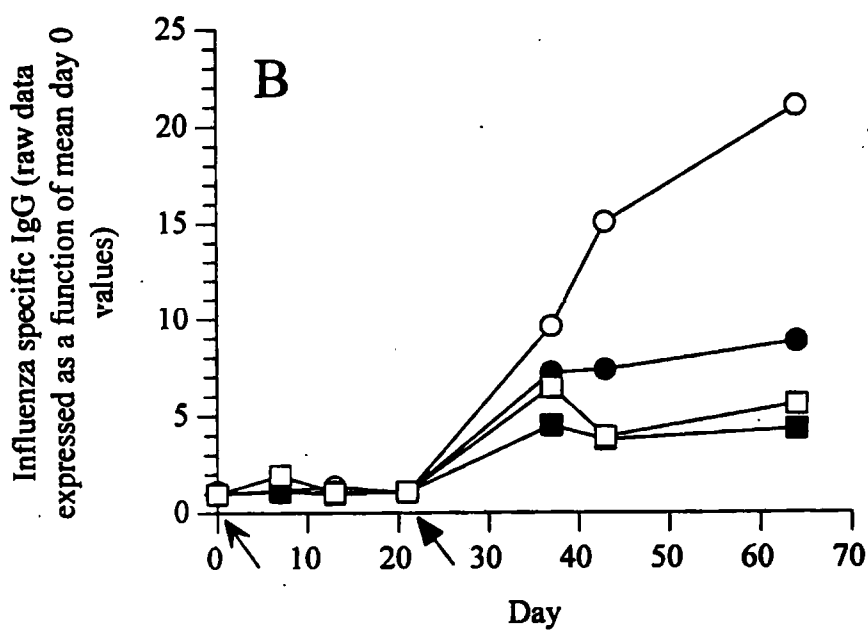
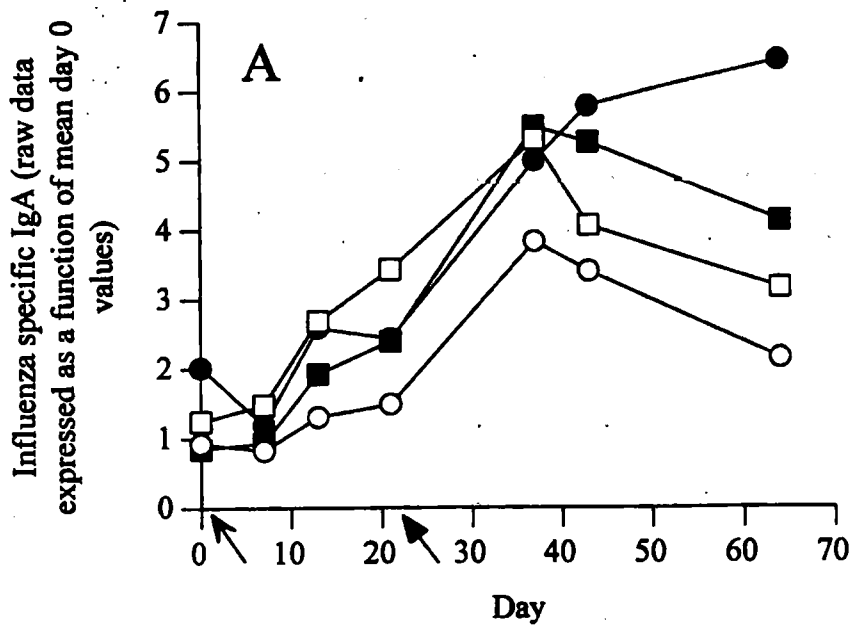


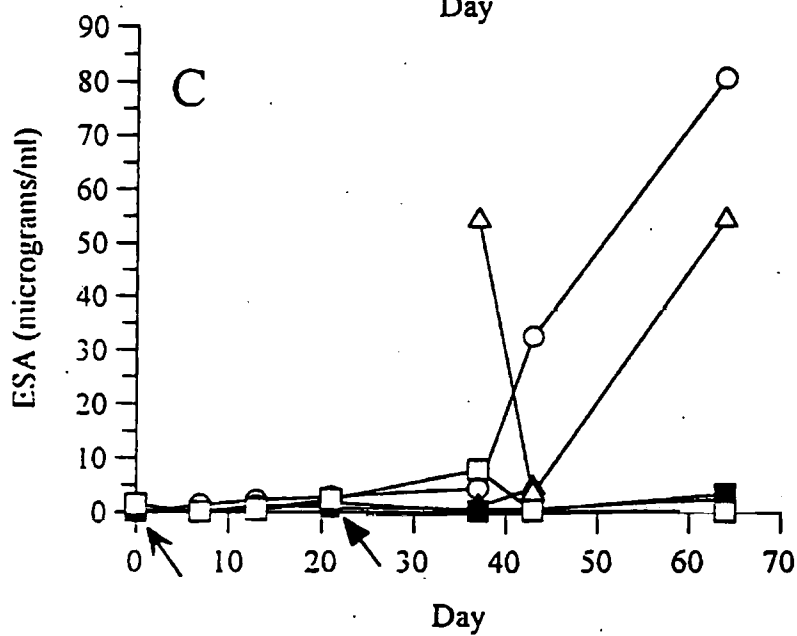
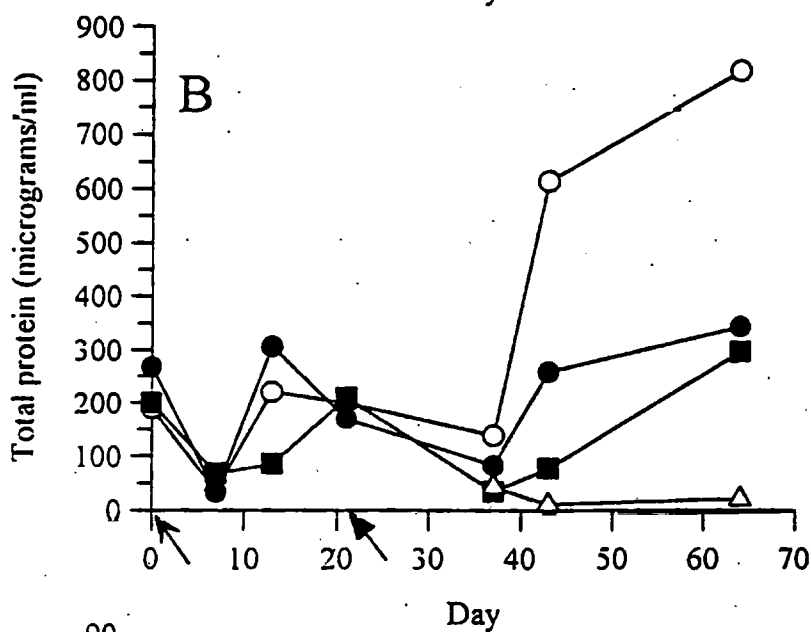
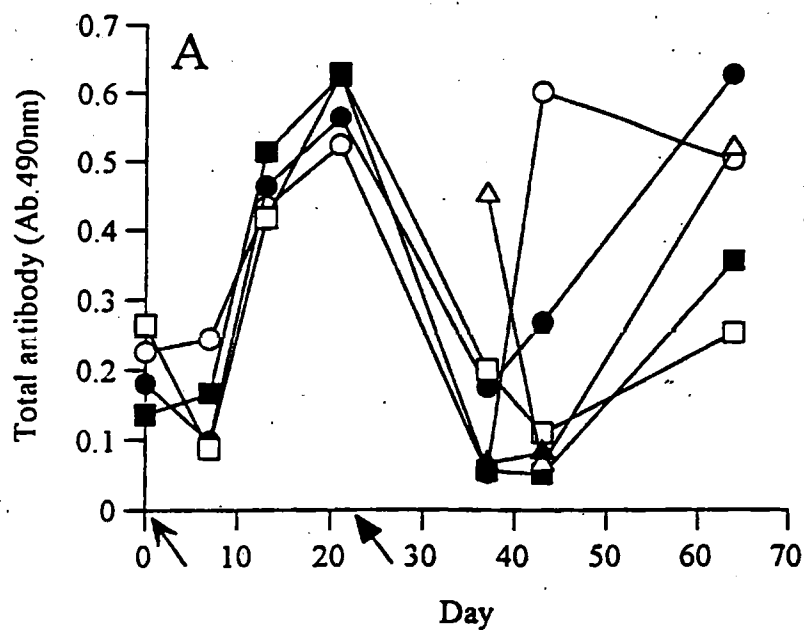
Figure 2.2 Flux of measured characteristics of nasal washes over the course of vaccination and infection.

Figure 2.2, graph A shows the levels of total immunoglobulin (Ab.490nm) found in nasal washes after vaccination (day 0, open arrow) and infection (day 22, closed arrow).

Graph B shows the concentration of protein ($\mu\text{g/ml}$) found in nasal washes after vaccination (day 0, open arrow) and infection (day 22, closed arrow).

Graph C shows the concentrations of equine serum albumin (ESA, $\mu\text{g/ml}$) found in nasal washes after vaccination (day 0, open arrow) and infection (day 22, closed arrow).

○ D70 ● 26E □ A58 ■ 001 △ P18 ▲ P16



values (ELISA data Ab.490nm from day $n \div$ ELISA Ab.490nm data from day 0; see figure 2.1).

Influenza specific antibody in BAL samples, taken 14 days after challenge, was measured by ELISA. The levels of ESA in these BAL samples were assayed and the influenza specific ELISA data divided by the concentrations of ESA, to give an arbitrary unit of influenza specific antibody per microgram ESA (data not shown). The ratio of influenza specific IgG:IgA in the BAL sample was significantly greater than that found in the nasal secretions ($p < 0.001$). The change in ratio was predominantly because of an increase in absolute levels of influenza specific IgG present in the BAL and a concomitant, but smaller decrease in influenza specific IgA. The mean ratio of influenza specific IgG:IgA in the BAL samples was 4.4 (SD = 0.987), and in the nasal wash 1.317 (SD = 0.46).

The amounts of total immunoglobulin (see 2.2.6), ESA (see 2.2.7), and total protein (see 2.2.11) were also determined (see figure 2.2). A discussion of the flux of total protein and ESA after vaccination and challenge can be found in 2.3.4 *Analysis of results*. Briefly, the levels of both total protein and ESA remained relatively constant over the course of vaccination, whereupon, large increases could be observed after infection, presumably because of epithelial pathological events.

Total levels of IgA and IgG were assayed by ELISA (for methods see 2.2.4 and 2.2.5, for results see 2.3.4 *Analysis of results* and figure 2.3). Briefly, vaccination and challenge had profound effects on the levels of total IgA and IgG present in the nasal washes. After vaccination there was a general increase in total levels of both isotypes and after challenge a transient reduction in total Ig-isotypes was observed.

2.3.2 *Virus neutralisation activity of nasal washes in tissue culture.*

The virus neutralisation activity of the nasal washes taken after vaccination and infection were determined (see 2.2.10). The results indicate that the intranasally vaccinated individuals (A58, D70 and 26E) had little or no virus neutralising antibody in their nasopharynx before challenge. Fifteen days after challenge, the amount of virus-neutralising antibody in nasal wash of A58 was similar to that found in the previously infected ponies, P16 and P18.

Table 2.1.1, Virus-neutralisation activity of nasal wash (expressed as a Neutralisation Index)

| Pony | Vaccinated | Day 0 | Challenge -1 | Challenge +15 | Challenge +42 |
|------|------------|-------|-----------------|------------------|------------------|
| A58 | + | 0 | ND | 2.25 | ND |
| D70 | + | 0.4 | -0.125 | 0.625 | ND |
| 26E | + | ND | 0.325 | 1.5 | 1.5 |
| P16 | - | ND | ND | 2.375 | ND |
| P18 | - | ND | ND | 2.625 | ND |

Footnote: Assays to measure Neutralisation indices (NI) of nasal washes were developed during the course of this experiment and were considered to be a measure of the biological function of the vaccine or infection induced nasopharyngeal antibody (NI = TCID₅₀ of a stock virus - apparent TCID₅₀ of the stock virus preincubated with nasal wash, for method see 2.2.10)

2.3.3 *Haemagglutination activity of nasal washes*

Nasal washes taken after infection were shown to inhibit virus-induced haemagglutination of chicken red blood cells. The HI titres expressed as log₂ showed a strong correlation ($r=0.8851$) with virus neutralisation activity *in vitro* (figure 2.4).

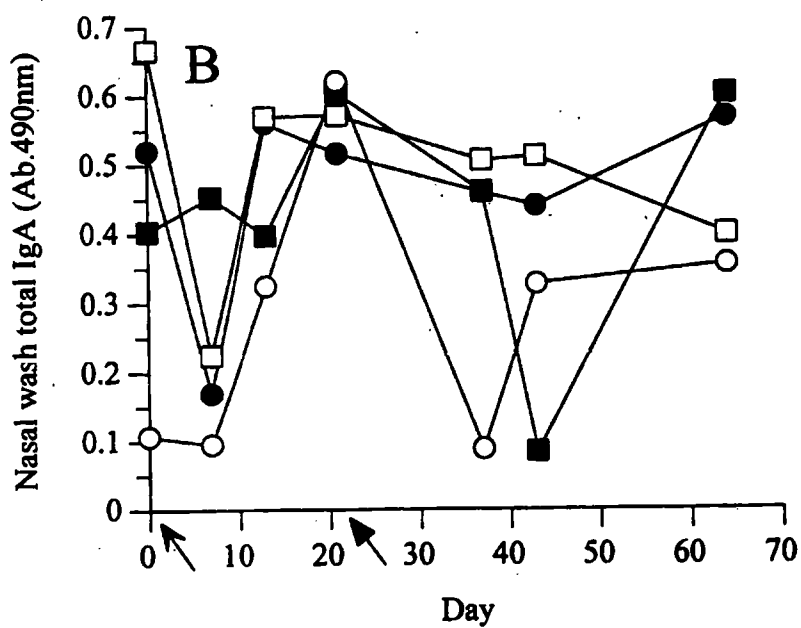
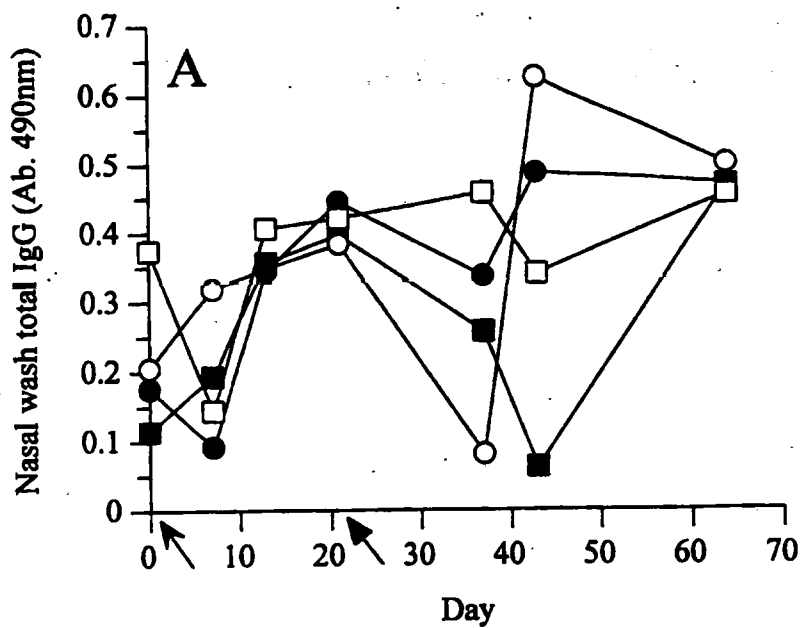
2.3.4 *Analysis of results section*

This study provides an excellent example of why the nasal wash standardisation techniques are crucial for accurate interpretation of nasal wash ELISA data. Examination of the raw nasal wash influenza specific ELISA data (figure 2.1) would suggest that the three ponies, that were vaccinated with CTB/S89, had detectable levels of influenza specific IgA at the time of challenge. Moreover, the pony 001, which had been vaccinated with CTB alone also had significant levels of influenza specific antibody in its nasal secretions. This evidence is contradictory to the observation that no VN antibody could be detected in these samples and the fact that all ponies became infected after experimental challenge. Ideally the parameter which is used to standardise the nasal wash data would be an easily measurable feature which remains relatively constant over the time course of an experiment, and whose flux are shared in both vaccinates and control animals.

Figure 2.3 Flux of isotype specific immunoglobulin in nasal washes over the course of vaccination and infection.

Figure 2.3 shows the levels of total IgA (graph A) and IgG (graph B) in nasal washes after vaccination (day 0, open arrow) and infection (day 22, closed arrow).

■ 001 ○ D70 ● 26E □ A58



In an attempt to negate the variability of nasal secretion dilution during the nasal wash procedure, and therefore its influence on the raw ELISA data, three methods of standardisation were used. These methods will be discussed, in turn, below.

A) *Standardisation using ESA*

A competition ELISA was developed to measure the concentrations of equine serum albumin (ESA) present in nasal washes (for method see 2.2.7). The concentration of ESA was determined for each nasal wash (figure 2.2, graph C). By dividing the raw influenza specific ELISA data by the concentration of ESA present in the nasal wash, an arbitrary unit for influenza specific antibody (Ab.490nm) per microgram ESA in the nasal wash was calculated (Figure 2.5).

The levels of ESA in nasal washes during the course of the vaccinations remained very low, at the limit of detection, in certain animals after infection, however, these levels increased dramatically (figure 2.2, graph C). This could be explained by the pathology of the influenza virus, causing damage of the epithelium of the nasopharynx, and thereby allowing ESA to leak directly from the circulation into the nasopharynx. Moreover, when the ESA data was used to standardise the influenza specific data from our four animals, the pony which had received CTB alone showed the highest level of influenza specific antibody of the group. This level was equivalent to that seen in the control animals 14 days after infection.

Therefore, where comparisons between nasal washes taken from protected and infected animals are necessary, the use of ESA as a parameter to standardise nasal washes would lead to inaccurate conclusions.

B) *Standardisation by protein concentration*

The protein concentration of each nasal wash was determined as described in 2.2.11 (figure 2.2, graph B). By dividing the raw influenza specific ELISA data by the protein concentration of each nasal wash, an arbitrary unit for influenza specific antibody (Ab.490nm) per microgram of total protein present in the nasal wash was calculated (figure 2.6).

The measurement of total protein in each nasal wash is very easy and may only suffer from the sensitivity of the Bio-Rad assay itself. The data shown in figure 2.2, graph B, show a large inter-pony variation. Once this data has been used to standardise the influenza specific ELISA data (figure 2.6), it is clear to see that according to this method no pony had any influenza specific immunoglobulin in their nasal secretions at the time of challenge.

The standardisation method using total ESA (see above), and total protein, do not take into account the fact that after challenge any leakage of systemic immunoglobulin into the nasopharynx would be of a predominantly IgG isotype. Any effect on the influenza specific IgA data would therefore be misleading.

To take one pony as an example, pony D70 had the largest increase in ESA after challenge. This pony also had the largest increase in total protein after challenge (figure 2.2). D70 also had a very large rise in total IgG present in the nasal wash (figure 2.3). However, the level of total IgA in the nasal wash did not increase so dramatically (figure 2.3). It seems, therefore, that standardisation of nasal wash influenza specific IgA data with the levels of total IgA would not be sensitive to leakage of systemic protein after challenge.

Figure 2.4 Relationship between nasal wash virus neutralisation (VN) and haemagglutination inhibition (HI) activity.

Figure 2.4 shows the correlation of VN and HI activity of nasal washes. Open circles represent an individual nasal wash taken before or after infection. r = correlation coefficient.

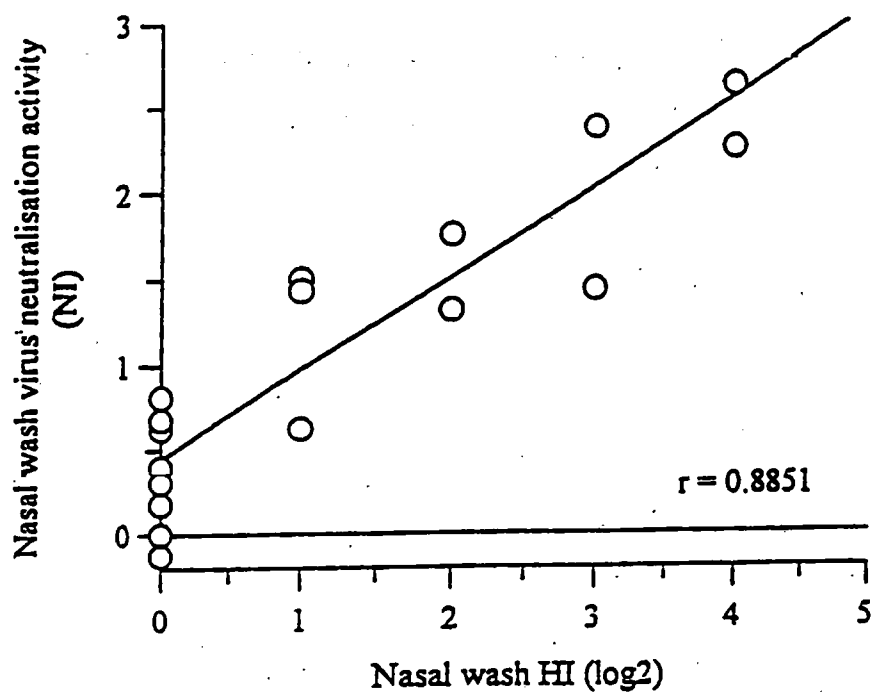


Figure 2.5 Influenza specific immunoglobulin in nasal washes expressed as a function of total equine serum albumin.

Figure 2.5 shows the changes in influenza specific IgA (graph A) and IgG (graph B) in nasal washes after vaccination (day 0, open arrow) and infection (day 22, closed arrow). Data expressed as raw influenza specific ELISA data (Ab.490nm) from a nasal wash ÷ concentration of ESA in the nasal wash ($\mu\text{g/ml}$).

○ D70 ● 26E □ A58 ■ 001 △ P18 ▲ P16

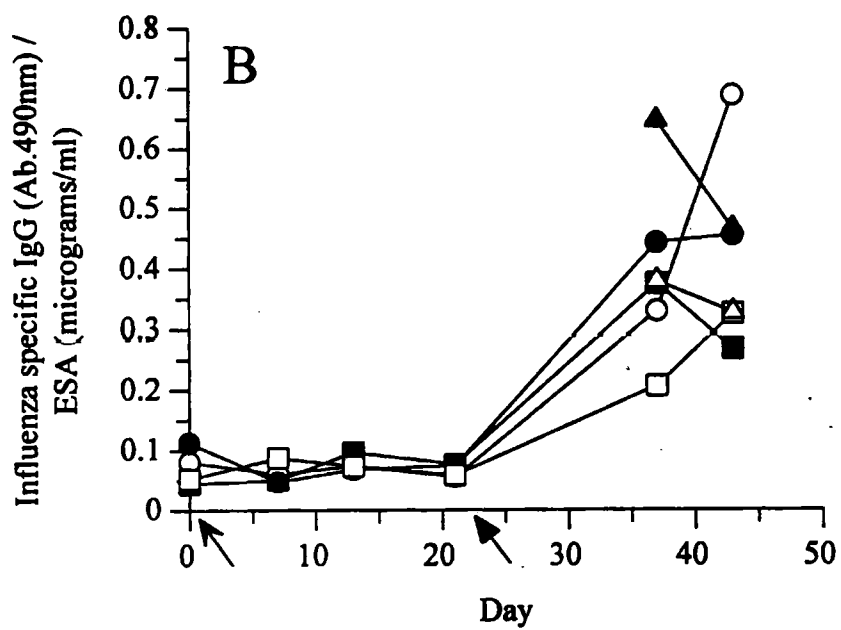
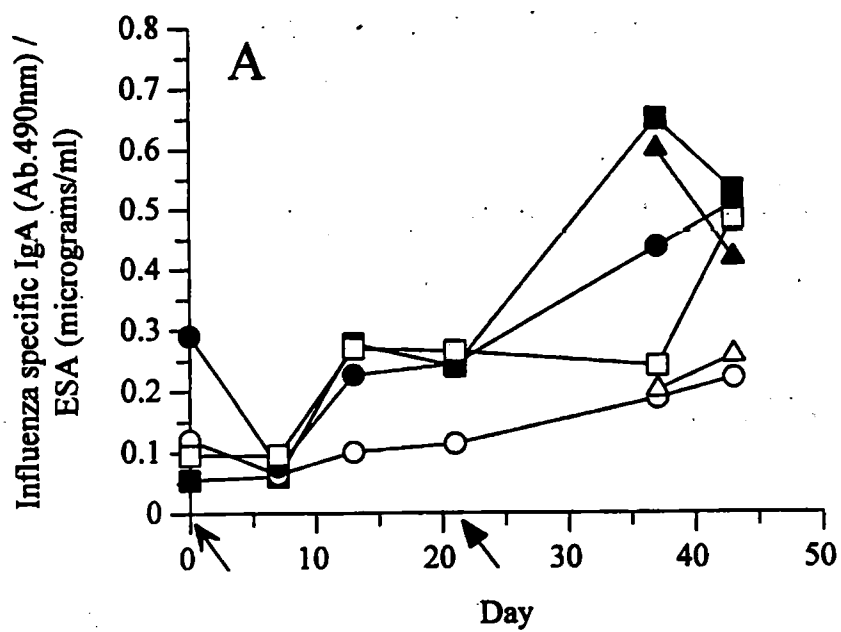


Figure 2.6 Influenza specific immunoglobulin in nasal washes expressed as a function of total protein.

Figure 2.6 shows the changes in influenza specific IgA (graph A) and IgG (graph B) in nasal washes after vaccination (day 0, open arrow) and infection (day 22, closed arrow). Data expressed as raw influenza specific ELISA data (Ab.490nm) from a nasal wash ÷ concentration of protein in the nasal wash ($\mu\text{g/ml}$).

■ 001 ○ D70 ● 26E □ A58

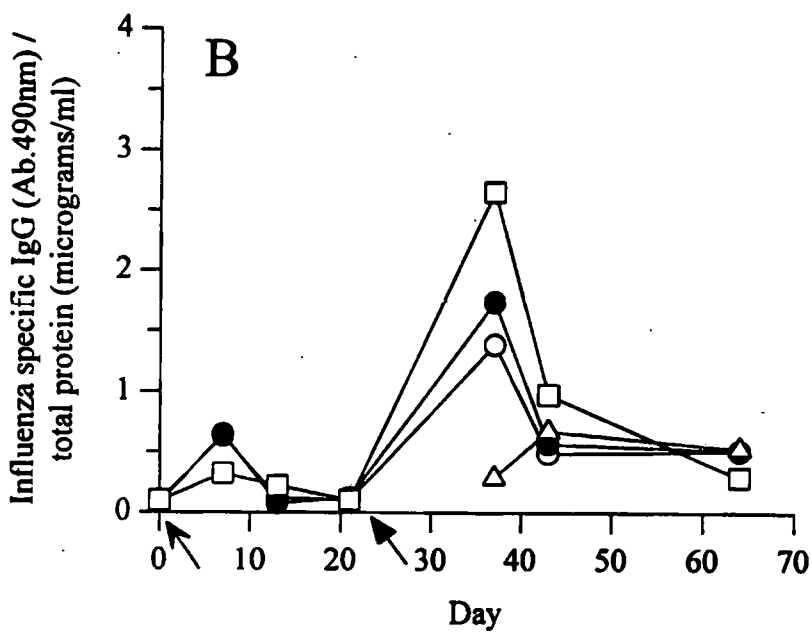
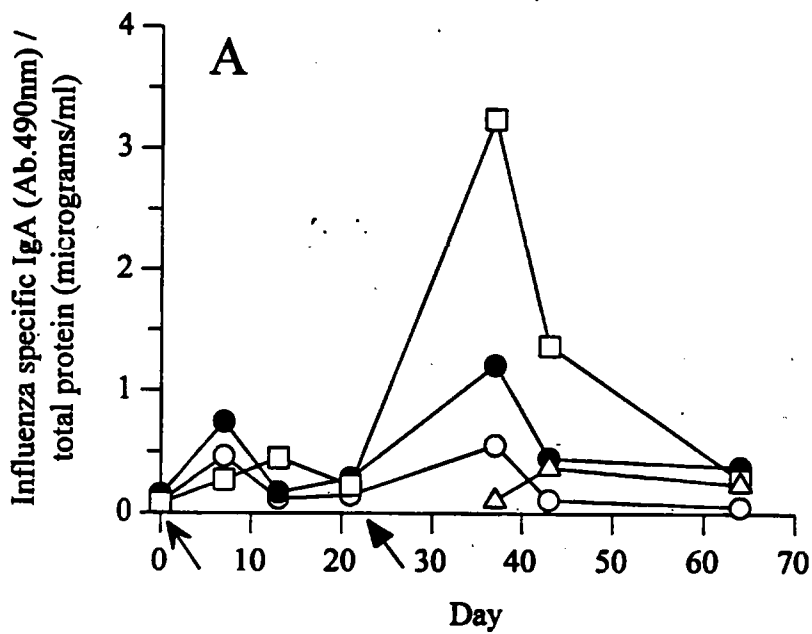


Figure 2.7 Influenza specific immunoglobulin in nasal washes expressed as a function of total isotype present in the nasal wash.

Figure 2.7 shows the changes in influenza specific IgA and IgG in nasal washes after vaccination (day 0, open arrow) and infection (day 22, closed arrow). Data expressed as raw influenza specific ELISA data (Ab.490nm) from a nasal wash \div total isotype present in the nasal wash (Ab.490nm).

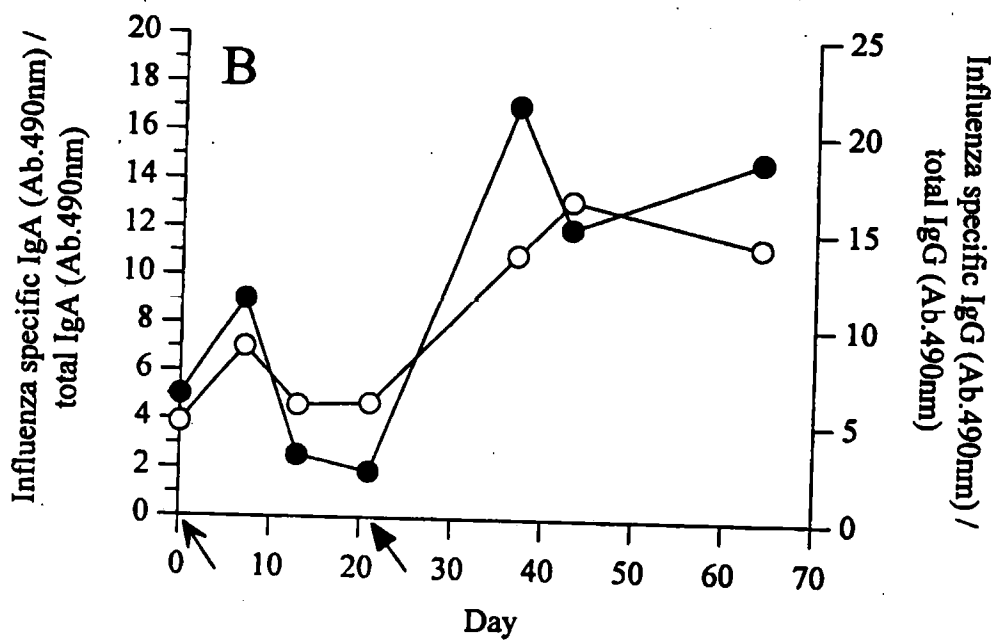
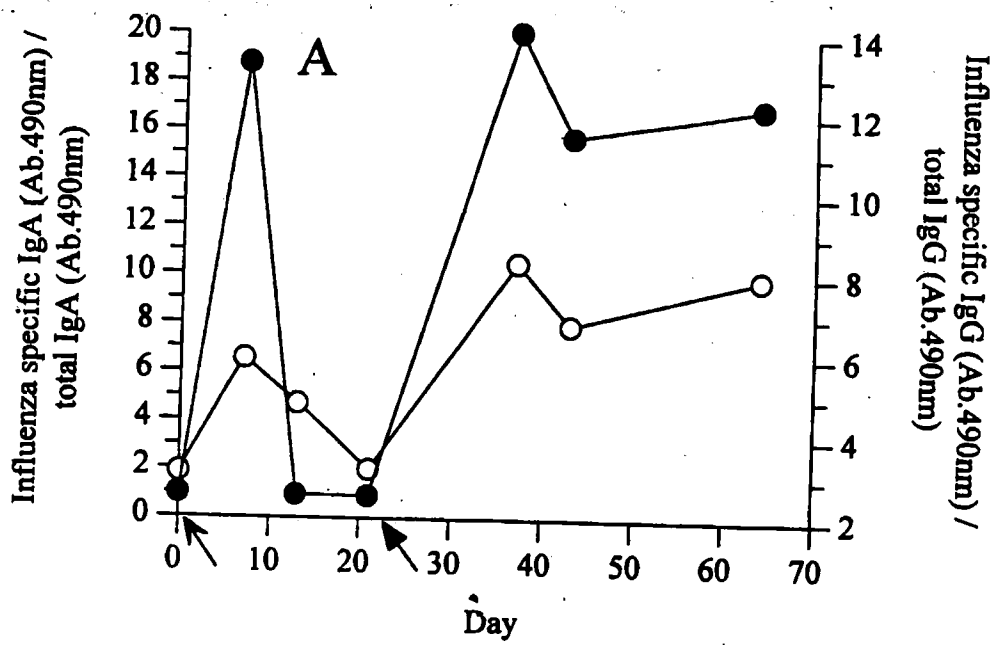
○— IgA ●— IgG

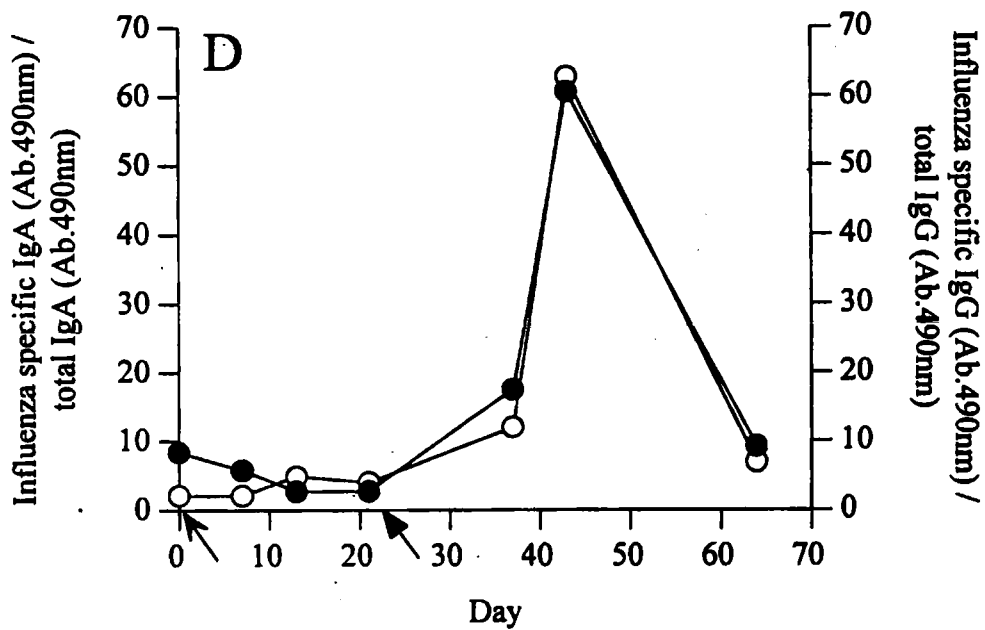
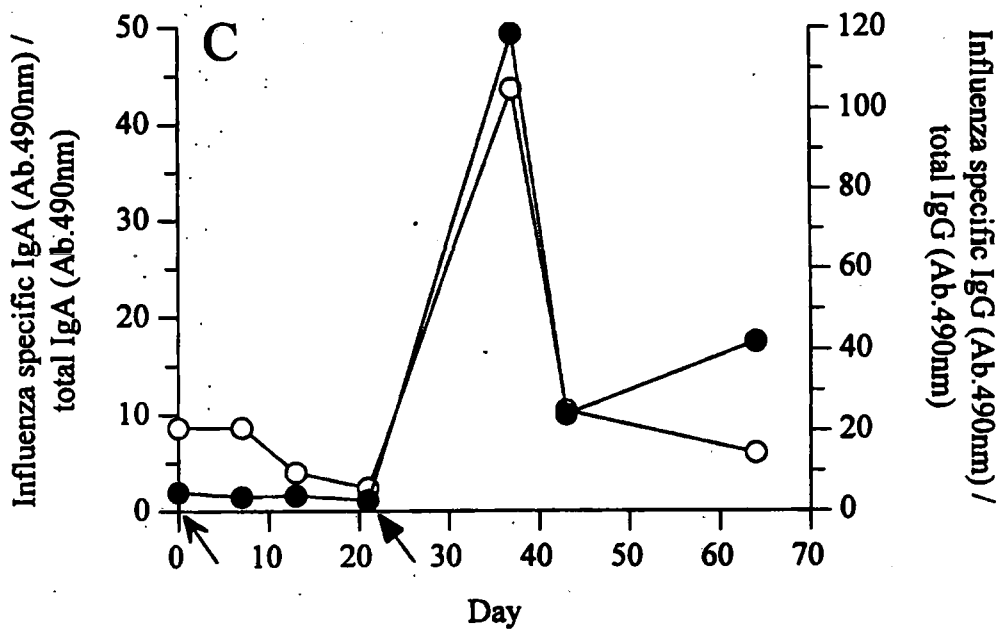
Graph A, pony A58

Graph B, pony 26E

Graph C, pony D70

Graph D, pony 001





C) Standardisation by total immunoglobulin isotype

A capture ELISA was also developed to measure levels of isotype specific total immunoglobulin in the nasal washes (figure 2.3), as described in 2.2.4 and 2.2.5. The raw influenza specific ELISA data (Ab.490nm) for each immunoglobulin isotype, was divided by the capture ELISA data (Ab.490nm) for the total levels of that isotype present in the nasal wash (see figure 2.7, graphs A to D). The resultant figure was an arbitrary unit which expressed the levels of influenza specific antibody in relation to the levels total nasal wash antibody.

Figure 2.7, graphs A to D, show the influenza specific IgA and IgG expressed as a function of total immunoglobulin isotype. The levels of IgA and IgG do not remain constant over the course of a vaccination/challenge experiment (figure 2.3) and as such their use to standardise the influenza specific data is always going to be a compromise. The main advantage that it does have, however, is the low intra-animal variation and the insensitivity of the total IgA levels to the influx of systemic protein after an experimental infection.

2.4 Summary

Techniques have been developed, as described in this chapter, to measure the antibody responses induced in nasal secretions by vaccination or infection. It has been shown to be vital, that influenza specific ELISA data is standardised to some definable parameter, in order that accurate interpretation is possible.

We have identified that influenza specific ELISA data is best expressed as a function of the total nasal wash antibody of that specific isotype (influenza specific ELISA data (Ab.490nm) / total isotype ELISA data (Ab.490nm)). This method was, therefore,

used during the remainder of this study.

Nasal wash immunoglobulin has been shown to have functional activity, and the methods to measure this have been developed. After infection, influenza specific antibodies show both haemagglutination inhibition and virus neutralisation activity *in vitro*.

Chapter 3

Measurement of B cell traffic

3. Measurement of B cell traffic

3.1.1 Introduction

During the early 1980's, the development of established solid phase immunoenzymatic assays allowed the enumeration of specific antibody secreting cells (Czerkinsky *et al.*, 1983; Sedgwick and Holt, 1983). This technique, termed ELISPOT, has developed through the years to include assays to measure not only antibody secreting cells (ASC) but also other cell secreted products such as cytokines (Hutchings *et al.*, 1989). In a manner similar to other enzyme-linked immunosorbant assays (ELISA) the ELISPOT is commonly performed in multi-well plates. By the use of an insoluble chromagen, the end result is an enumeration of cells that secrete a specific product rather than a measure of the concentration of the product (see figure 3.1 for schematic presentation of the technique).

3.1.2 *The use of ELISPOT to measure mucosal immunity*

Amongst its many uses, this technique has been utilised to measure the stimulation of the common mucosal immune system (CMIS, see chapter 1). According to the hypothesis of the CMIS, after a mucosal application of an antigen a stimulation of local B cells will occur. Clonal proliferation of these cells will take place in the local lymph nodes before entering the blood stream to traffic to distant mucosal effector sites (for extensive review see Phillips-Quagliata and Lamm, 1994). After mucosal vaccination or infection, ELISPOT assays are used to detect the appearance of antigen specific, antibody secreting B cells within the peripheral blood mononuclear cells (PBMC).

Circumstantial evidence to support the association between the trafficking ASC and the CMIS is the apparent temporal relationship between the appearance of antigen specific ASC in the blood, their later arrival at other distant effector sites, and the appearance of antigen specific immunoglobulins in the external secretions (Czerkinsky *et al.*, 1991; Moldoveanu *et al.*, 1995). However, the first solid evidence has only recently become available and this evidence revolved around the study of homing receptor expression on the surface of the ASC. Address markers dedicated to mucosal sites have been identified. In man one such receptor is MAdCAM-1, with its corresponding lymphocyte-expressed ligand, the integrin $\alpha 4\beta 7$ (Kilshaw and Murant, 1991; Hu *et al.*, 1992; Berlin *et al.*, 1993; Erle *et al.*, 1994; Hamann *et al.*, 1994; Viney *et al.*, 1996). The integrin $\alpha 4\beta 7$ has been shown to mediate binding to Peyer's patch high endothelial venules (HEV), but not peripheral lymph node HEV (Berlin *et al.*, 1993).

Quiding *et al.* (1995) demonstrated differences in the ASC responses derived from oral vaccination with cholera toxin B chain (CTB), and those derived from a systemic vaccination with tetanus toxoid (TT). Almost all of the TT specific ASC expressed L-selectin, a marker mediating homing to peripheral lymph nodes (Kushimoto *et al.*, 1990; Kansas, 1992), whereas only 40% of CTB specific ASC expressed this receptor. Kantele *et al.* (1996) further observed differences in patients with ongoing gastrointestinal infection. They observed an increased expression of $\alpha 4\beta 7$ on peripheral blood pathogen-specific ASC compared to the ASC of healthy controls, and a concomitant reduction in L-selectin expression.

3.1.3 *The application of ELISPOT assays after vaccination and infection*

ELISPOT assays have been utilised by many authors to investigate a wide variety of diseases and vaccines (for a recent review see Arvilommi, 1996). After an infection the

ELISPOT assay typically reveals pathogen-specific ASC in the peripheral blood from days 4-5, peaking at around day 7, and disappearing in around 2-4 weeks (Arvilommi, 1996). The ASC generally disappear from the blood only after the disappearance of the pathogen and the resolution of clinical signs.

Peripheral blood ASC have been reported after bacterial gastrointestinal, urinogenitory and respiratory infections (respectively: Kantele *et al.*, 1988; Kantele *et al.*, 1994; Nieminen *et al.*, 1996). Few studies have investigated the ASC responses after viral infections. Mäkelä *et al.* (1995) investigated army volunteers with acute upper respiratory tract infections including adeno- or influenza viruses. ASC specific for either pathogen could be detected by ELISPOT in the blood of the patient at clinical presentation. The numbers of ASC rapidly declined over time and were down to almost undetectable levels by 4 weeks after initial presentation. It is interesting to note that IgG was the dominant isotype of immunoglobulin to be produced, and that the numbers of ASC were not demonstrably linked to the titres of virus specific IgG in the convalescent serum.

According to its status as the most vigorously investigated mucosal adjuvant, cholera toxin (CT) has been used by many authors to investigate B cell responses after mucosal vaccination. In both mice and man, ASC responses are induced after oral or intranasal application of CT (Chen and Strober, 1990; Lewis *et al.*, 1991; Jertborn *et al.*, 1994; Quiding *et al.*, 1995; Czerkinsky *et al.*, 1991). These ASC appear in the peripheral blood of humans 7 days after oral vaccination, with roughly equal frequencies of IgG and IgA secreting cells (around 80 ASC/ 10^6 PBMC, Quiding *et al.*, 1995).

ASC specific for both CT and bystander antigens have been detected in peripheral blood after oral (Jackson *et al.*, 1993; Quiding *et al.*, 1995) and intranasal (Wu and Russell, 1993; Russell *et al.*, 1996) application of the CT adjuvanted vaccine. CT specific ASC

derived from oral vaccination of humans exhibit "mucosal" B cell phenotypes, and therefore favour the population of mucosal effector sites (Quiding *et al.*, 1995). Russell and colleagues (1996) provided further evidence for the mucosal "destiny" of circulating ASC by inducing antigen specific IgA in mucosal secretions in the absence of IgA in the serum. Intranasal vaccination of rhesus monkeys with CTB together with bacterial antigen (Ag I/II), induces Ag I/II specific antibodies in serum, saliva, faecal and genital samples. ASC specific for Ag I/II, mainly of the IgA isotype, were found in the peripheral blood 1 week after vaccination. The resulting antibodies, IgA and IgG, present in nasal and vaginal washes may not have been derived from the same cellular source. Nasal IgG showed a strong correlation with serum IgG, and therefore may have been derived by transudation from the circulation. In contrast, nasal IgA, and both vaginal IgA and IgG, did not correlate with plasma antibody responses, suggesting a contribution of local synthesis (Russell *et al.*, 1996).

The experimental evidence supports the thesis that ASC derived by mucosal vaccination express homing receptors favouring the mucosae. Also, antigen specific immunoglobulins may be induced in external secretions in the absence of antigen specific antibody in the circulation. Taken together these findings support the idea that circulating ASC observed after mucosal vaccination reflect the induction of mucosal immunity.

The aim of this study is to develop an ELISPOT assay for use in the equine system and apply it to the study of B cell responses after mucosal vaccination and infection. This technique will be used to verify immunological stimulation, and by implication provide evidence for the existence of a CMIS in the horse.

3.2 Materials and Methods

3.2.1 Animal Work

Intranasal vaccination

It was previously determined that horses tolerated an intranasal application of CTB (100 µg [Sigma cat.no.C-9903]), in terms of absence of pyrexia and other clinical signs. Ponies that were to receive the vaccine were sedated using 0.2 ml of intravenous Domosedan [Veterinary drug company]. 50 µg of CTB was instilled into both nasal passages using either of the two techniques described in chapter 4. Rectal temperatures were taken, the ponies were kept in boxes and observed at regular time periods up to 24 hours after application. Ponies received two applications of CTB, one "priming" dose and another 3-4 weeks later. Over the time course of the experiment heparinised blood samples (for ELISPOT assay, see 3.2.2), serum and nasal wash (see 2.2.1) samples were taken for later analysis.

Influenza challenge

Ponies with low, or absent, serum influenza specific serum antibody (HI) were selected to go onto influenza challenge. These animals were experimentally infected by exposure to infectious aerosol as described by Mumford *et al.* (1990). Briefly, after an isolation of influenza virus from a field outbreak of equine influenza in 1989, A/Equi/2/Sussex/89 virus, as it became known, was passaged thrice in fertile hens eggs, as described in 5.1.2.1. This large stock of challenge virus was diluted from 10^0 to 10^{-6} , and used in an experimental infection, wherein nebulised virus was introduced to groups of ponies in a sealed challenge room, to determine the minimum infectious dose (MID). Subsequent

experimental infections were performed using $10^4 \times \text{MID}$ of the frozen stock of challenge virus, in the same challenge room.

Blood samples were taken by jugular venipuncture and nasal wash samples were taken (see 2.2.1) during the course of the infection.

3.2.2 Isolation of PBMC

Blood samples were collected from ponies by jugular venipuncture into 15 ml vacutainers containing 225 units of sodium heparin ((Mucous) Heparin injection BP, [CP Pharmaceuticals, Wrexham, UK]). The red blood cells were allowed to settle and the PBMC rich plasma was loaded onto Ficoll-Paque [*Pharmacia Biotech AB, Uppsala, Sweden, cat No. 17-0840-03*] for density gradient centrifugation (30 min @ 275g, 22°C). PBMC were harvested from the interface between the plasma and Ficoll-Paque, and were washed twice, by centrifugation and resuspension (10 min @ 275g) in Hanks wash (Hanks balanced salt solution, without calcium or magnesium [*ICN Flow, cat. no. 18-104-54*], containing 200 IU/ml penicillin [*ICN Flow cat. no. 16-702-49*], 200 µg/ml streptomycin [*ICN Flow, cat. no. 16-702-49*], and 5ml of a 7.5 % sodium bicarbonate solution [*ICN Flow, cat. no. 16-883-49*]). The PBMC were resuspended in complete RPMI (RPMI 1640 [*ICN Flow, cat. no. 12-602-54*] containing 10% heat inactivated foetal calf serum [*Sigma cat. no. F-2442*], 2 mM/ml L-glutamine [*ICN Flow, cat. no. 10-801-49*], 100 IU/ml penicillin, 100 µg/ml streptomycin (cRPMI)), and were counted using a haemocytometer. The cell concentration was adjusted to 4×10^6 PBMC/ml cRPMI.

3.2.3 ELISPOT assay

24 well tissue culture plates [*Costar, Cambridge MA 02140, cat.no. 3524*] were coated overnight, @ 4°C, with antigen (either cholera toxin B chain [*Sigma, cat.no. C9903*] or influenza virus (see chapter 5)) diluted to 50 µg/ml in 0.05M coating buffer (carbonate-bicarbonate buffer pH9.6, [*Sigma, cat.no. C-3041*]). The plates were incubated for 1 hr @ 37 °C in blocking buffer (phosphate buffered saline [*Unipath Ltd, cat.no. BR14*], containing 0.05% Tween 20 [*Sigma, cat.no. P-1379*], and 2% dried skimmed milk [*Biorad, cat.no. 170-6404*]) to block all free sites. The plates were washed thrice with washing buffer (phosphate buffered saline [*Unipath Ltd, cat.no. BR14*], containing 0.05% Tween 20 [*Sigma, cat.no. P-1379*]).

Cells were added, in triplicate, in 250 µl volumes. Each well contained either 5×10^5 or 1×10^6 PBMC. The plates were incubated overnight @ 37°C, 5% CO₂. The plates were then washed vigorously with tap water and then thrice with washing buffer. The primary antibody was then added, affinity purified γ -chain specific anti-equine IgG-biotin (1:500) [*Dynatech, K&P, cat.no. 16-21-02*] or monoclonal anti-equine IgA-biotin (K129-3E7, kindly donated by Professor Chris Stokes), 1:500) diluted in blocking buffer, and incubated for 1 ½ hours @ 37°C. The plates were washed thrice with washing buffer. Streptavidin-alkaline phosphatase (1:1000) [*Sigma, cat.no. E-2636*] diluted in blocking buffer was added and incubated for 1 hour @ 37°C. The plates were washed thrice in washing buffer. The insoluble chromagen 5-bromo-4-chloro-indolyl phosphate (BCIP [*Sigma, cat.no. B1026*], dissolved in AMP buffer (see appendix B) @ 100 mg/ml) was added and incubated @ 37°C until the spots became visible. The reaction was stopped with distilled water and the plates left to dry. The spots were enumerated using a stereo dissection microscope.

3.2.4 ELISA assays for antibody quantification

ELISA assays were used to follow CTB and influenza specific immunoglobulins in either serum or nasal wash samples. Methods to determine total isotype specific immunoglobulin present in nasal wash samples are given in 2.2.4 and 2.2.5. The influenza specific ELISA for both nasal wash and serum antibody were performed as described in 2.2.3.

Immunoglobulins present in serum and nasal washes specific for CTB were followed by ELISA. 96-well flat bottomed ELISA plates (Immunlon 3 [Dynatech cat.no. 011-010-3755]) were coated with 20 µg/ml CTB [Sigma cat.no. C-9903] diluted in coating buffer, and incubated overnight @ 4°C. All additions were in 100 µl volumes. The plate was incubated with blocking buffer for 1 hour @ 37°C. The plates were washed thrice with washing buffer. Serum samples were added at a dilution of 1:50 and titrated two-fold across the plate. Nasal wash samples were added neat and titrated two-fold across the plate. The samples were incubated for 1 hour @ 37°C. The plates were washed thrice in washing buffer and the anti-immunoglobulins were added, affinity purified γ-chain specific anti-equine IgG-biotin (1:1000) [Dynatech, K&P, cat.no. 16-21-02] or monoclonal anti-equine IgA-biotin (clone 3E7, 1:500) diluted in blocking buffer, and were incubated for 1 hour @ 37°C. The plates were washed thrice in washing buffer and streptavidin-horseradish peroxidase, (1:1000 [Amersham, cat.no. RPN 1231]) diluted in blocking buffer, was added and incubated for 1 hour @ 37°C. The plates were washed thrice with washing buffer and the chromagen O-phenyldiamine (OPD [Sigma, cat.no. P6912]) diluted to 0.5 mg/ml in phosphate-citrate buffer, with sodium perborate [Sigma, cat.no. P-4922], was added. After 20 minutes for the colour to develop the reaction was stopped, with 50 µl/well 4M H₂SO₄, and the absorbance read at 490 nm (Dynatech ELISA plate reader, DIAS system).

Figure 3.1 Schematic representation of the ELISPOT technique.

- a) Multi-well plates are coated with antigen.
- b) A predetermined number of peripheral blood mononuclear cells (PBMC) are added to the wells and incubated overnight, 4°C.
- c) The PBMC settle to the bottom of the well where they secrete their specific antibodies. Antigen specific immunoglobulins bind to the plate in the vicinity of their cell of origin.
- d) All PBMC and non-specific immunoglobulins are washed off.
- e) Anti-immunoglobulin conjugates are added and therefore mark the position of the antigen specific cell.
- f) A chromagen is added and its insoluble product forms a spot which can be counted using a stereomicroscope.

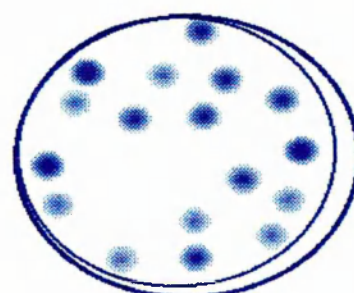


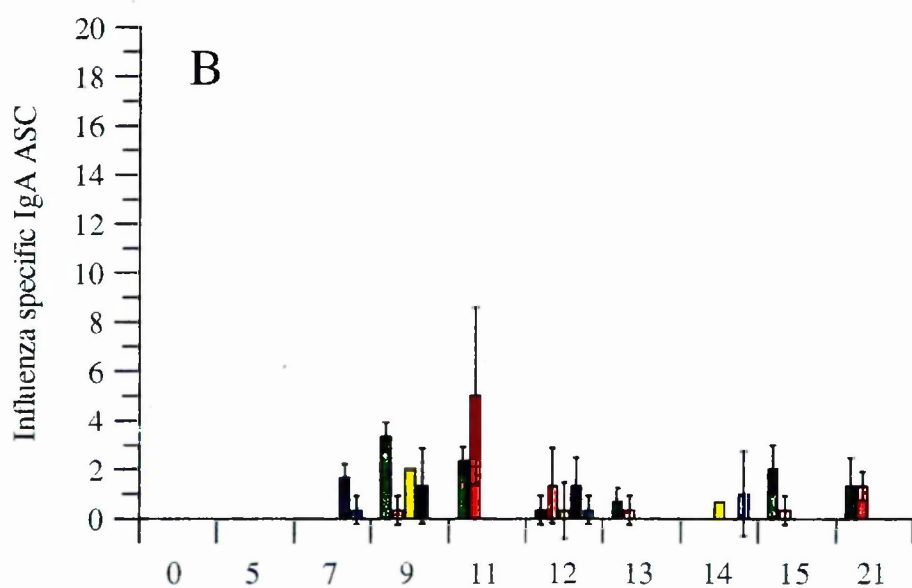
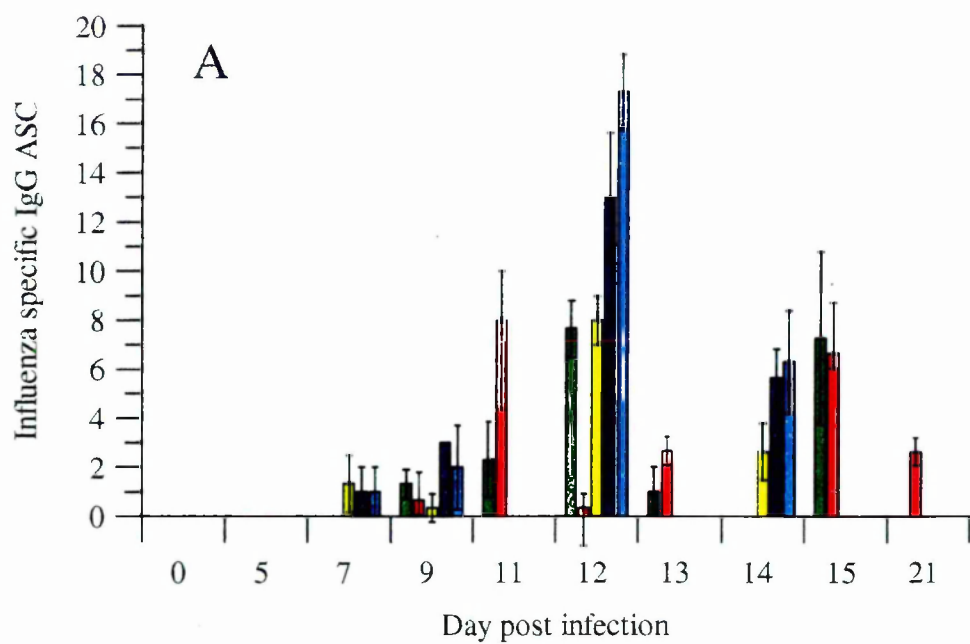
Figure 3.2 **Influenza specific ASC in the PBMC of ponies after experimental infection.**

Figure 3.2 shows the numbers of influenza specific ASC in the circulation of five ponies after an experimental influenza infection (on day 0). Graph A shows the numbers of IgG producing ASC and Graph B shows the numbers of IgA producing ASC. Results are expressed as number of influenza specific ASC per 10⁶ PBMC. Nb, not all ponies were sampled at each time point:

| <i>Ponies</i> | <i>Sample schedule (day post infection)</i> |
|---------------|---|
| 11, 14 and 15 | 0, 7, 9, 12, 14 |
| 001 and P18 | 0, 5, 7, 9, 11, 12, 13, 15, 21 |

Ponies:

001 P18 11 14 15



3.3 Results

3.3.1 *Trafficking B cells after experimental influenza infection*

ELISPOT assays were performed at various time points (up to 21 days) after an experimental influenza infection. All animals were considered "flu free" in terms of lack of influenza history (infection or vaccination), and absence of serum influenza specific antibody. The animals were challenged with influenza by aerosol on day 0. ASC specific for influenza appeared in the peripheral blood by day 7 after infection, peaked at around day 12, and then slowly declined by day 21 after infection (figure 3.2). ASC producing IgG were predominant with respect to IgA, showing a ratio of approximately 4:1. It should be noted that because of different ponies being challenged at different days not all animals were sampled on all of the time points shown in figure 3.2. This graph should be taken to show a general trend indicating the temporal appearance of influenza specific ASC in the peripheral blood of infected ponies.

3.3.2 *Trafficking B cells after intranasal vaccination with CTB*

Three ponies received two intranasal applications of CTB. The ASC responses in the peripheral blood were followed and related to the temporal appearance of CTB specific immunoglobulins in nasal secretions and serum. This vaccination protocol resulted in high levels of CTB specific antibody in nasal secretions (IgA) and serum (IgG) (figure 3.3). Very few CTB specific ASC appeared in the peripheral blood after the first priming dose. However, significant numbers of CTB specific ASC appeared 5 days after the intranasal boost. The numbers of ASC peaked at around day 8, and declined to low levels by day 21 after boosting. Again IgG was the predominant immunoglobulin isotype produced.

The appearance of CTB specific IgA ASC in the peripheral blood coincided with the appearance of CTB specific IgA in the nasal secretions. Very little CTB specific IgG

Figure 3.3 Cholera toxin specific antibody responses in nasal wash and serum of one pony, after intranasal vaccination with cholera toxin B chain (CTB).

Figure 3.3 shows changes in CTB specific IgA and IgG in nasal wash (graph A), expressed as arbitrary units (raw CTB specific data Ab.490nm / total isotype Ab.490nm). Graph B, CTB specific IgA and IgG in serum expressed as raw ELISA data (Ab.490nm). Intranasal vaccination (with 100µg CTB, with the nasooaryngeal applicator) was performed on days 0 and day 30 (open arrows).

—○— IgA —●— IgG

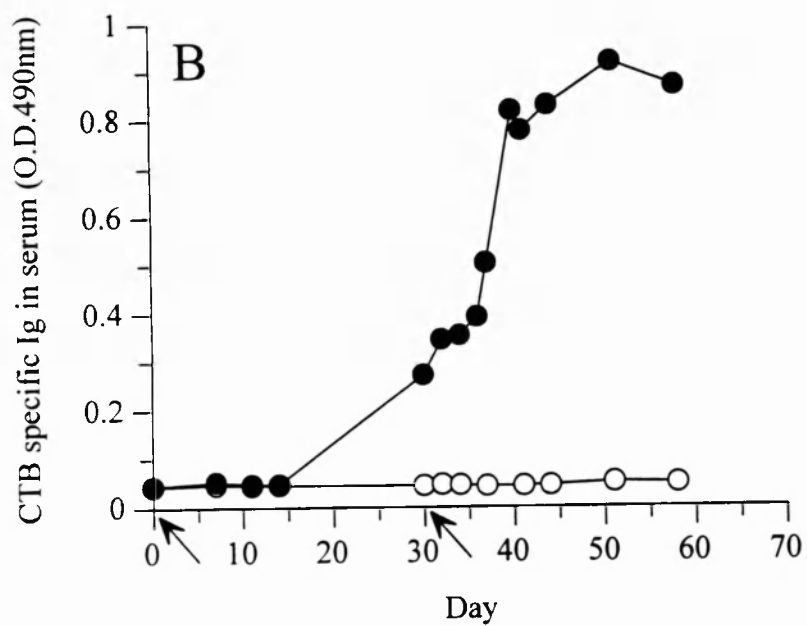
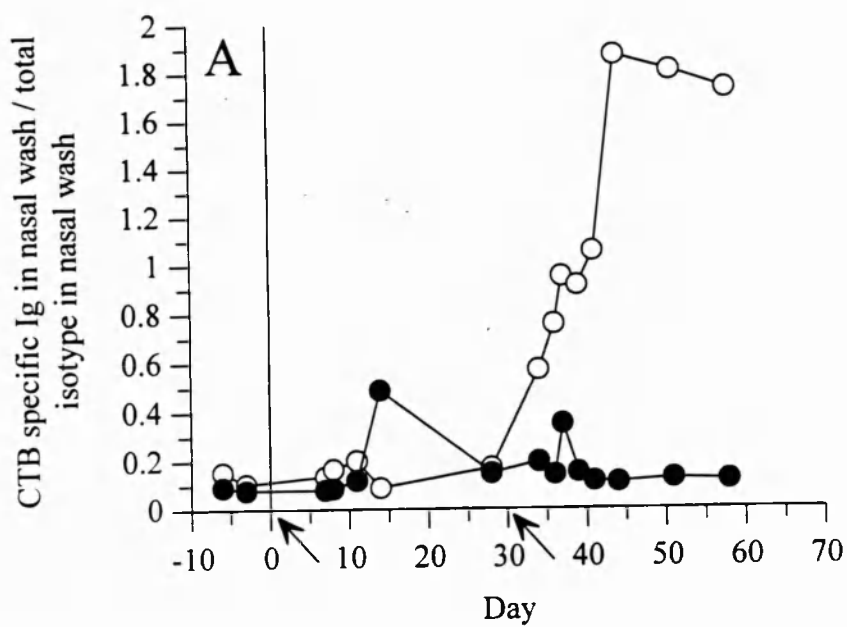


Figure 3.4 Relationship between the appearance of CTB specific ASC in the peripheral blood and the appearance of CTB specific immunoglobulin in nasal washes.

Figure 3.4, shows the temporal relationship between the appearance of CTB specific ASC in the peripheral blood and the appearance of CTB specific IgA and IgG in nasal washes after an intranasal boosting application of CTB. Peak numbers of CTB specific ASC occurred on days 7 and 8. The histograms are shown overlaying one another to emphasise the transient nature of the appearance of ASC in the peripheral blood.

Graph A, The appearance of CTB specific IgA ASC in the peripheral blood is represented by the histograms (showing the numbers of ASC/ 10^6 PBMC, on the right hand axis), and the changes in CTB specific IgA in nasal washes (Ab.490nm \div total IgA, on the left hand axis).

Graph B, The appearance of CTB specific IgG ASC in the peripheral blood is represented by the histograms (showing numbers of ASC/ 10^6 PBMC, on the right hand axis), and the changes in CTB specific IgG in nasal washes (Ab.490nm \div total IgG, on the left hand axis).

—●— D33 —●— E49 —●— 001 ■ 001 ASC ■ E49 ASC ■ D33 ASC

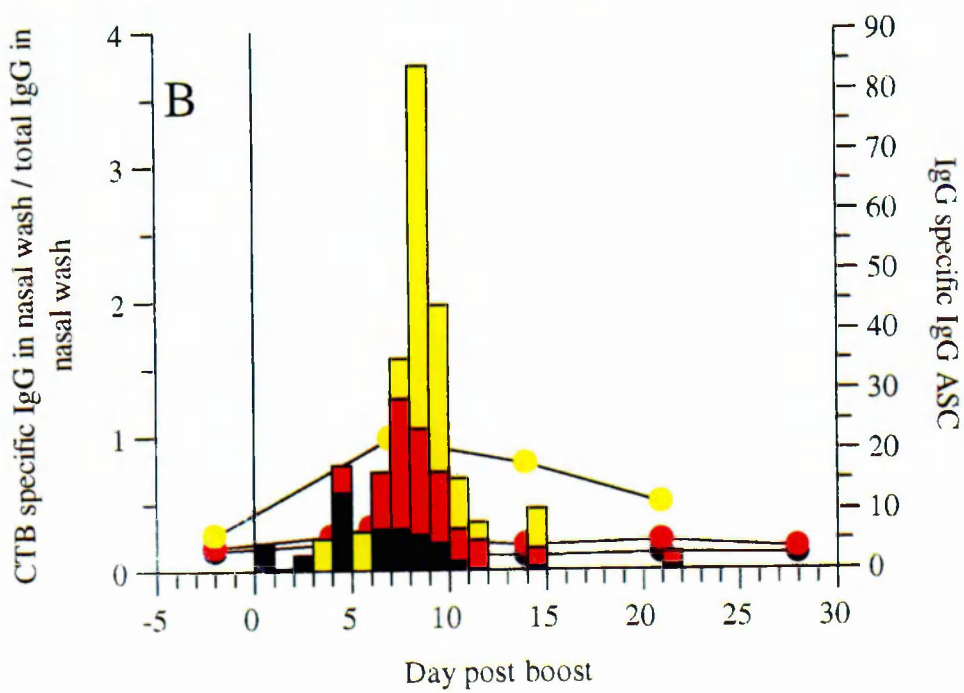
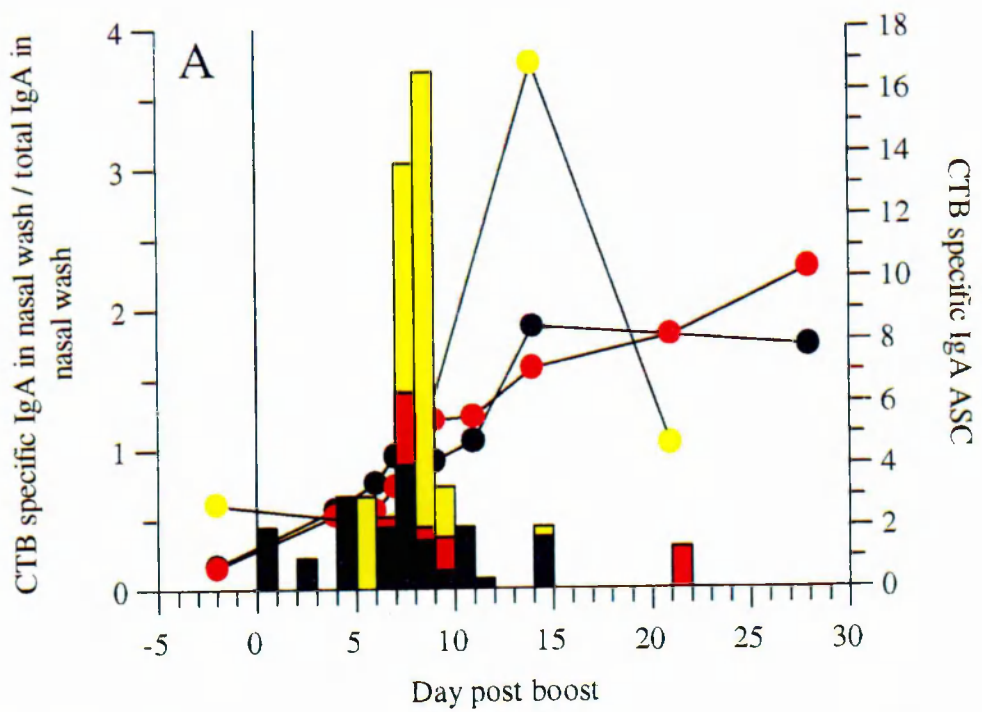


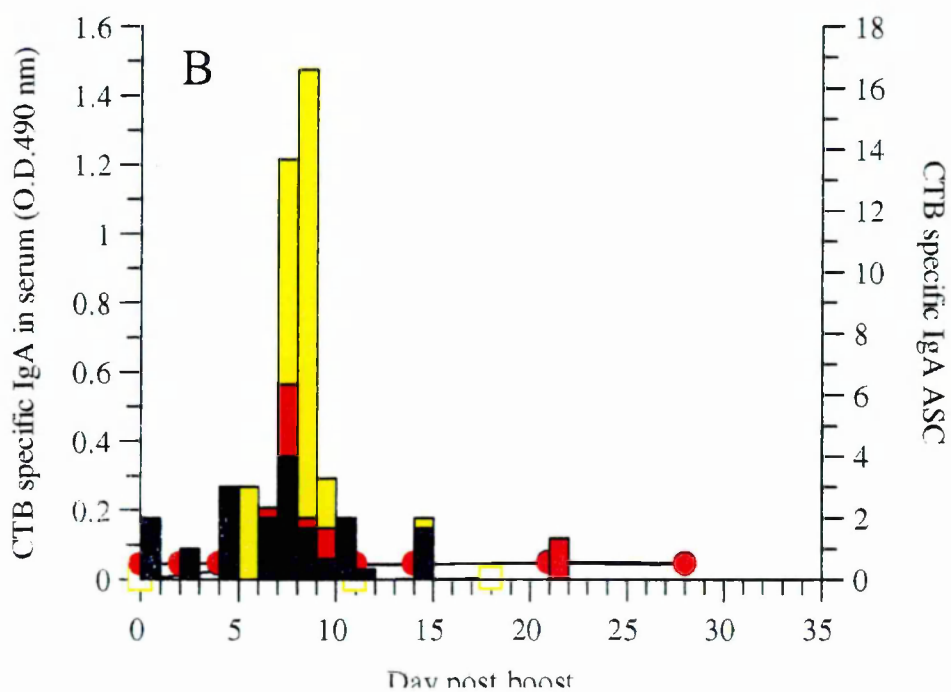
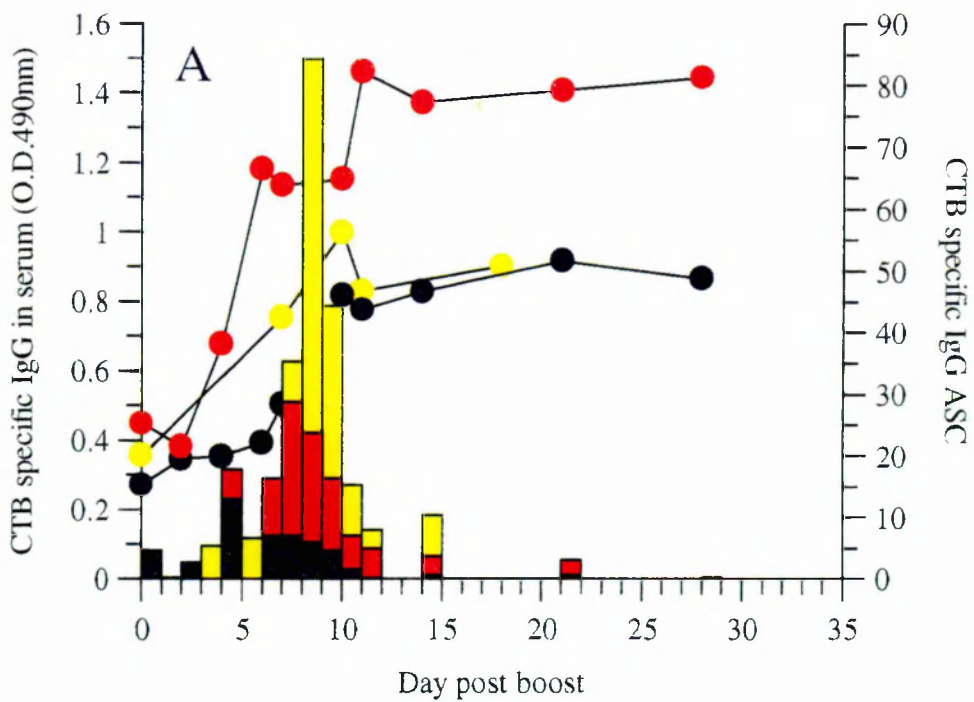
Figure 3.5 Relationship between the appearance of CTB specific ASC in the peripheral blood and the appearance of CTB specific immunoglobulin in serum.

Figure 3.5 shows the temporal relationship between the appearance of CTB specific ASC in the peripheral blood and the appearance of CTB specific IgA and IgG in serum after an intranasal boosting application of CTB. The peak of CTB specific ASC occurred on days 7 and 8. The histograms are shown overlaying one another to emphasise the transient nature of the appearance of ASC in the peripheral blood.

Graph A, The appearance of CTB specific IgG ASC in the peripheral blood is represented by the histograms (showing numbers of CTB specific ASC/ 10^6 PBMC, right hand axis), and the changes in CTB specific IgG in serum (Ab.490nm, left hand axis).

Graph B, The appearance of CTB specific IgA ASC in the peripheral blood is represented by the histograms (showing numbers of CTB specific ASC/ 10^6 PBMC, right hand axis), and the changes in CTB specific IgA in serum (Ab.490nm, left hand axis).

—●— 001 —●— D33 —●— E49 □ 001 ASC ■ E49 ASC ■ D33 ASC



appeared in the nasal secretions despite very high numbers of CTB specific IgG ASC in the peripheral blood (figure 3.4). In contrast the appearance of CTB specific IgG ASC coincided with a sharp rise in CTB specific IgG in serum. No CTB specific IgA was found in the serum despite significant numbers of CTB specific IgA ASC in the PBMC (figure 3.5).

3.4 Discussion

3.4.1 *ASC responses in the peripheral blood after influenza infection*

In common with other species, ASC specific for influenza virus have been detected in the peripheral blood of horses after infection. The numbers of ASC, and the isotype secretion profile, are in accord with those found after a human influenza infection (Mäkelä *et al.*, 1995).

ELISA assays of nasal wash samples taken from horses after infection revealed that the ratio of influenza specific IgG (ELISA O.D.), to IgA (ELISA O.D.) was about 1. The ratio of IgG to IgA in bronchoalveolar lavage fluid (BALF), on the other hand, was about 4:1 (see chapter 2). Therefore the ratio of influenza specific IgG : IgA in the BAL more closely reflected the ASC situation in the peripheral blood than that found in the upper respiratory tract.

The ratio of IgG : IgA producing plasma cells varies throughout the length of the equine respiratory tract, IgA predominates in the upper respiratory tract while IgG predominates in the lower airways (Mair *et al.*, 1988). It is therefore interesting to note that even though a large proportion of the total influenza specific ASC in the peripheral blood produce IgG,

50% of the nasopharyngeal influenza specific antibody is of the IgA isotype. Conversely, in another mucosal effector site (*ie*, the lower airways) there is a similar proportion of IgG : IgA to that found in the peripheral blood ASC. It should be noted, however, that the lack of influenza specific IgG in the nasopharynx may be because of the non-existence of a transport pathway for locally produced IgG to enter the airway lumen, and not the fact that IgG producing ASC do not selectively locate to the URT. Furthermore, these conclusions depend on the hypothesis that the influenza specific IgG present in the BAL samples is locally produced and not simply a transudate directly from the peripheral blood.

These observations suggest the existence of a system of selective B-cell trafficking, whereby mucosally activated B-cells producing influenza specific IgA selectively populate the respiratory tract of the horse.

3.4.2 ASC responses in the peripheral blood after intranasal application of CTB

CTB specific ASC were detected in the peripheral blood of horses after intranasal vaccination. This finding is in agreement with other authors working in other species (see introduction 3.1.2).

As in the experiments following ASC after influenza infection, the immune responses after intranasal vaccination with CTB showed a stark difference between IgA and IgG responses. The data suggest that the IgA ASC detected in the peripheral blood, are selectively locating to the mucosal surfaces. The IgA appearing in nasal secretions is therefore most likely to be locally produced. The IgG ASC, on the other hand, seem to be restricted to producing their product in the serum only, or mucosal sites other than the

nasopharynx. It will be interesting to investigate this further in order to elucidate the homing receptor expression on trafficking equine lymphocytes.

Intranasal vaccination with CTB induces trafficking IgA producing ASC which seem to be populating exclusively mucosal effector sites. The appearance of these ASC in the peripheral blood precedes, or coincides with, the appearance of CTB specific antibodies in external secretions. The results of this study provide the first description of antigen specific ACS responses after mucosal vaccination in the horse. These observations support the hypothesis of the existence of a CMIS in the horse, and also emphasises the utility of ELISPOT assays to measure the stimulation of the mucosal immune system after mucosal vaccination. This method should have application for furthering research knowledge on the trafficking signals for equine lymphocytes.

Chapter 4

Development of the nasopharyngeal
applicator

4. **Development of a nasopharyngeal applicator.**

4.1 **Introduction**

Many studies have been reported of intranasal vaccination regimes which purport to stimulate mucosal immune responses in the nasopharynx and/or the upper respiratory tract. It is common practice to vaccinate mice intranasally by instillation of relatively large volumes of liquid vaccine and on occasions this has resulted in asphyxiation (C. Elson, personal communication). Ideally, an intranasal vaccine should be administered in a small volume and make contact with all the putative mucosal induction sites, preferably in the form of a spray of fine droplets. Groups working in the human field have looked at efficient nasal spray applicators (Kuno-Sakai *et al.*, 1994; Hashigucci *et al.*, 1996). Fortunately, the horse has large nostrils and the nasopharynx is easily accessible for the purpose of vaccination and nasal wash sampling.

Work by Tim Mair (1987) described the distribution of lymphoid follicles, the putative induction sites of the mucosal immune response, within the respiratory tract of the horse. In brief, he showed isolated single follicles throughout the upper respiratory tract (URT) with large aggregations of follicles in the pharyngeal recess, nasopharynx (especially around the entrance to the auditory tube), and around the larynx. A mucosal vaccine in the horse must therefore be targetted towards these putative induction sites in order to attain the most efficient uptake of antigen.

Simple flexible plastic pasteur pipettes have been used in horses to instill infectious virus into the nostrils of horses (Hannant *et al.*, 1993), and these were used as a method for intranasal vaccination at the beginning of this study. However, liquid was expelled from these pipettes as a jet rather than a spray and it was thought that maximum coverage of the

nasopharynx would be difficult with this system. Therefore, a more targeted delivery system was devised which not only delivered the vaccine directly into the nasopharynx but also increased the coverage area.

4.2 Materials and Methods

4.2.1 *Use of the pastuer pipette*

The vaccine (approximately 500 μ l) was sucked all the way into the bulb of a flexible fine tipped pasteur pipette (127-P406-000 Elkay Eireann, Costelloe, Co. Galway, Ireland). The end of the pipette was inserted 10 cm up one nostril of the horse (*i.e.* the entire length of the pipette, not including the bulb), and the bulb was squeezed in rapid succession until the whole volume had been instilled.

4.2.2 *Construction and use of the nasopharyngeal applicator*

The basis of the applicator is a spray-unit dissected from a ladies hairspray can. The spray-unit can be used from any aerosol based on a "rocker" type rather than a simple "button" type spray. By the use of this type of aerosol, the spray-unit can be carved to have a long thin tube. An experimental system was constructed by wedging the spray unit into the end of a short length (approximately 40cm) of clear PVC tubing (Portex, 6.5mm external diameter \times 2.5 mm internal diameter). A one-way stopcock, fitted with male and female luer adaptors (Vygon 871.10, Benkat Instruments, Ilkeston), was attached to the other end of the tube (figure 4.1).

Figure 4.1 The nasopharyngeal applicator.

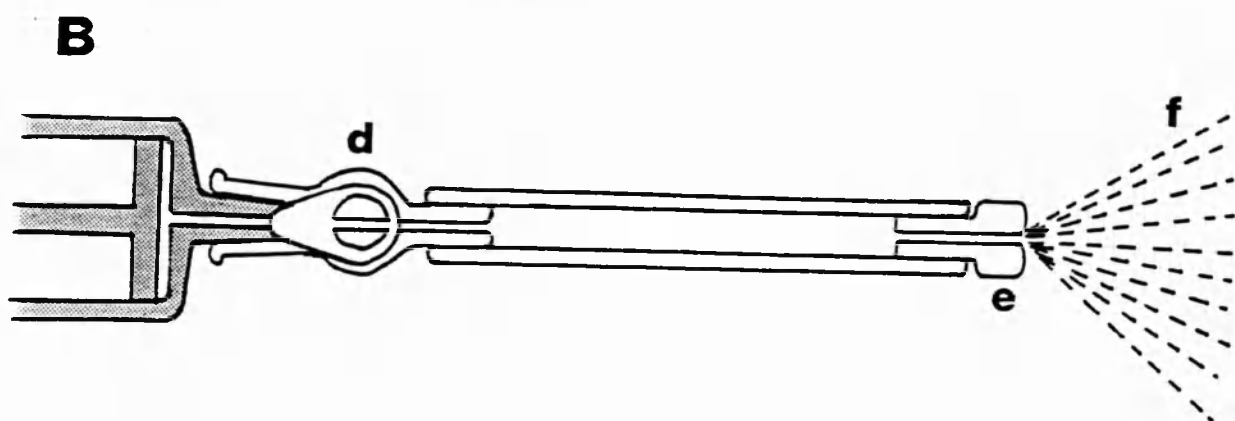
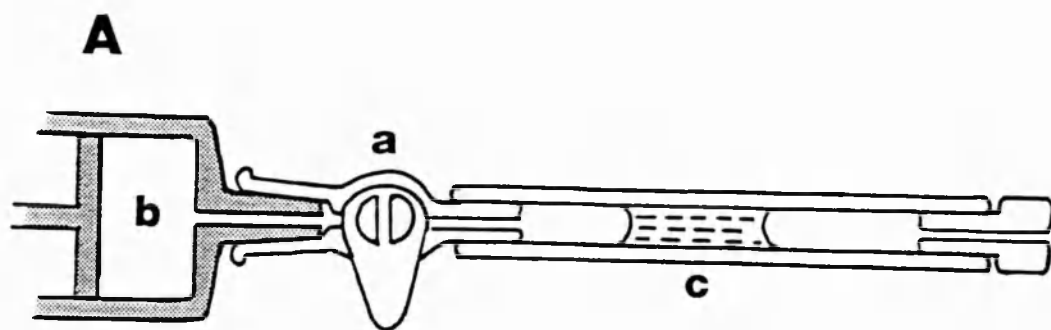
Figure 4.1 shows the construction of the nasopharyngeal applicator. *nb.* the length of the tubing is not to scale.

A *Preparation before use.*

With the gas tap turned off (a) the syringe is compressed to create a reservoir of air under pressure (b). The vaccine is held within the tubing (c).

B *Use of nasopharyngeal applicator.*

When the gas tap is turned to its on position (d) the compressed air is released and the vaccine is forced through the spray nozzle (e) and deposited into the nasopharynx in a mist (f).



To use the applicator, the spray-unit end of the applicator was held upright and a small volume of vaccine was instilled into the tube through the stopcock. The stopcock was then turned to its "off" position. A 50 ml syringe, filled with air, was inserted into the female luer adapter of the stopcock and the applicator was then inserted into the nostril of the horse to the desired location (see below). By squeezing the syringe a small volume of air under pressure was created, which was released by opening the stopcock. This compressed air forced the vaccine through the spray-unit end of the tube. The stopcock was then turned "off" and a second cycle of 50ml of air was performed to discharge the residual vaccine left in the tube.

By examination of postmortem material it was found that the best location to release the vaccine was at the distal part of the nasal cavity at the opening of the nasopharynx. The length that the applicator had to be inserted into the nasal cavity was calculated to be $4/5 \times$ the distance between the opening of the false nostril and the medial canthus of the eye.

4.2.3 *Comparison of old v.s. new methods*

A comparison of the old pasteur pipette and new nasopharyngeal applicator was performed on two ponies which were to be euthanased for reasons unconnected with this study. 1% methyl violet aqueous solution was instilled into the nares of the animals ante-mortem, in order to observe the areas covered by each vaccination method. In one pony, two applications (each of 500 μ l) of 1% methyl violet was squirted up each nostril using the pasteur pipette. Likewise, in another pony 2 \times 500 μ l of 1% methyl violet was sprayed into the nasal passages using the nasopharyngeal applicator. The ponies were then euthanased, the heads were removed and split para-sagittally to one side of the nasal septum

using a bandsaw (Biro, model 3336). The nasal septum was removed from the bisected head and the mucosal surfaces were inspected for traces of dye.

4.3 Results

Pasteur Pipette method

Inspection of the mucosal surfaces revealed a stream of dye along the ventral nasal meatus and along the floor of the nasopharynx. A very small amount of dye was observed on the lateral edges of the epiglottis. No staining was observed at the entrance to the auditory tube or in the nasopharyngeal recess. A small quantity of dye flowed from the nares of the pony (figure 4.2).

Nasopharyngeal applicator method

After bisection, complete staining of the distal 3-5 cm of the ventral meatus was observed. There was complete staining of the entire nasopharynx, epiglottis and proximal arytenoid, with a little dye reaching the proximal laryngeal canal. Small spray droplets were seen on the proximal dorsal, middle, and ventral meatus. Dye completely covered the auditory tube opening and nasopharyngeal recess (figure 4.2).

No traces of dye were observed in the oropharyngeal tonsil or esophagus using either method.

Figure 4.2 A comparison of two methods of intranasal vaccination.

Figure 4.2 A, shows the extent of dye coverage when applied with the pasteur pipette method (as described in 4.3).

Figure 4.2 B, shows the extent of dye coverage when applied with the novel nasopharyngeal applicator (as described in 4.3)



A



B

4.4 Conclusion

It was concluded that the nasopharyngeal applicator gave not only a significantly greater area of coverage compared to the old pasteur pipette method, but also enhanced the targeting of the vaccine towards the putative induction sites. The acceptability of this method of vaccination was confirmed by demonstrating the apparatus to an equine practitioner (Mr Robert Allpress). The applicator was used successfully in non-halter-broken ponies without the use of systemic sedation and Mr Allpress agreed that the system could be used in practise.

Chapter 5

Experimental chapter

Chapter 5. Experimental chapter

5.1 Comparison of vaccine preparations

5.1.1 Introduction

The realisation that CTB could be used as an adjuvant as an alternative to the toxic holotoxin stimulated many authors to construct various vaccine combinations which have met varying success. It has been used admixed with (Tamura *et al.*, 1988b; Russell *et al.*, 1996), chemically (Liang *et al.*, 1989; Wu and Russell, 1993 and 1995; Russell *et al.*, 1996) or genetically (Hajishengallis *et al.*, 1995) conjugated to the vaccine antigen.

Chemical conjugation of antigen to cholera toxin affects the antigenicity of both components, and the GM1 binding activity of the CTB (Liang *et al.*, 1989). It was decided, therefore, to carry out a pilot study comparing the antigenic and immunogenic properties of 3 vaccine formulations, namely CTB/influenza conjugated using glutaraldehyde, CTB/influenza conjugated using 3-(2-pyridyldithio) propionic acid succinimide ester (SPDP), and a simple mixture of CTB with influenza whole virus. These formulations were to be used to vaccinate three groups of ponies, each group containing naive and systemically primed animals, and the immunological responses assessed.

5.1.2 Materials and methods

5.1.2.1 Virus antigen preparation

Influenza virus (A/equi/2/Sussex/89) was grown in the allantoic cavity of 10 day old embryonated hens' eggs for 72 hours at 34°C. The eggs were placed at 4°C overnight prior

to harvesting. The allantoic fluid was clarified by low speed centrifugation ($1000 \times g$, 4°C) followed by high speed centrifugation to pellet the virus (2.5 hours at average $g = 29,500$, 4°C). The resuspended pellet was then further purified by centrifugation on a discontinuous (15%, 30%, 60%) sucrose gradient at 4°C for 1.5 hours at average $g = 51,850$. Virus was collected from the 30%/60% interface and washed in sterile PBS. Protein concentration of the resulting virus stock was determined using the Micro BCA protein assay [Pierce, cat. no. 23235] after boiling for 4 minutes in PBS containing 1% SDS. Prior to use in the vaccine the correct concentration of virus stock was heat inactivated (h.i.) at 56°C for 1.5 hours. The h.i. virus was then passaged twice in 10 day old fertile hens eggs to verify the inactivation of the virus.

5.1.2.2 Conjugation of CTB and influenza virus with glutaraldehyde

A/Equi/2/Sussex/89 influenza virus was adjusted to 2 mg/ml in PBS (containing 1mM EDTA [Sigma, cat. no. E-9884]). An equal volume of the cross-linking reagent glutaraldehyde (40 mM, EM grade [Sigma, cat. no. G5882]), in PBS (containing 1mM EDTA) was added and mixed for 18 hours at room temperature. The reaction mixture was dialysed against 2000 volumes of PBS (containing 1mM EDTA). Cholera toxin B chain [Sigma, cat. no. C9903] was added at a ratio of 1:25 w/w, and incubated for 24 hours at 4°C . The conjugate was then dialysed against 2000 volumes of PBS (containing 1mM EDTA), and D-lysine [Sigma, cat. no. L-5501] was added to a final concentration of 20 mM. Conjugation of the proteins was evidenced by ELISA tests using GM1 ganglioside to capture the conjugate and revealed by anti-influenza antibody. The final product was dialysed for 2 hours against 2000 volumes of PBS in a dialysis tubing with a 30,000 MW cut-off membrane, to purify the conjugate.

5.1.2.3 Conjugation of CTB and influenza virus with SPDP

The conjugation procedure was performed with 3-(2-pyridyldithio) propionic acid succinimide ester (SPDP) which incorporates a disulfide bridge between the two conjugated species, following manufacturers instructions [*Pierce, cat. no. 21557*]. Briefly, the two proteins were diluted to 2 mg/ml in borate buffered saline (BBS [*Pierce, cat. no. 28384*]), and both separately treated with the SPDP stock reagent for 30 minutes at room temperature. The proteins were dialysed against 2000 volumes of BBS to remove excess reagent. The SPDP-CTB protein was reduced with dithiothreitol (DTT), and the SPDP derivatised influenza protein was mixed with the DTT-reduced CTB for 18 hours at room temperature. The conjugate was separated from low molecular reagents such as free SPDP and DTT by size exclusion chromatography on a Sephacryl® S-300 column. Evidence for conjugation was assessed as described in 5.1.2.2. The final product was dialysed for 2 hours against 2000 volumes of PBS in a dialysis tubing with a 30,000 MW cut-off membrane, to purify the conjugate.

5.1.2.4 Animals and vaccination schedules

Three Welsh mountain ponies which had been infected 6 months previously, with A/equi/2/Sussex/89, and three naive 6 month old foals were used for intranasal vaccination. Three groups, each containing one naive foal and one previously infected pony, were vaccinated intranasally with either CTB/S89 mixture (containing 100µg CTB and 300µg heat inactivated S89; S89/CTB mix.) , CTB/S89 conjugated with glutaraldehyde (containing 300µg S89 and 12µg CTB, S89/CTB Glut.), or CTB/S89

conjugated with SPDP (containing 300µg S89 and 150µg CTB, S89/CTB SPDP). The doses of S89 and CTB used in the two conjugates as described above were determined from the starting amounts used in the conjugation reaction procedure, and not from a product of the reaction procedure. For a summary of the vaccination protocols see table 5.1.1. The vaccines were administered on two occasions with an interval of 4 weeks between the vaccinations.

Table 5.1.1, Immunological status of ponies and vaccine formulations

| Immunological state of the ponies | Vaccine formulation | | |
|--------------------------------------|---------------------|--------------|-------------|
| | S89/CTB Glut. | S89/CTB SPDP | S89/CTB mix |
| Naive | D70 | A58 | 26E |
| Previously infected | A | C | D |

Nasal wash and serum samples were taken periodically in order to follow the mucosal and systemic antibody responses.

5.1.3 Results

5.1.3.1 Nasopharyngeal influenza specific antibody

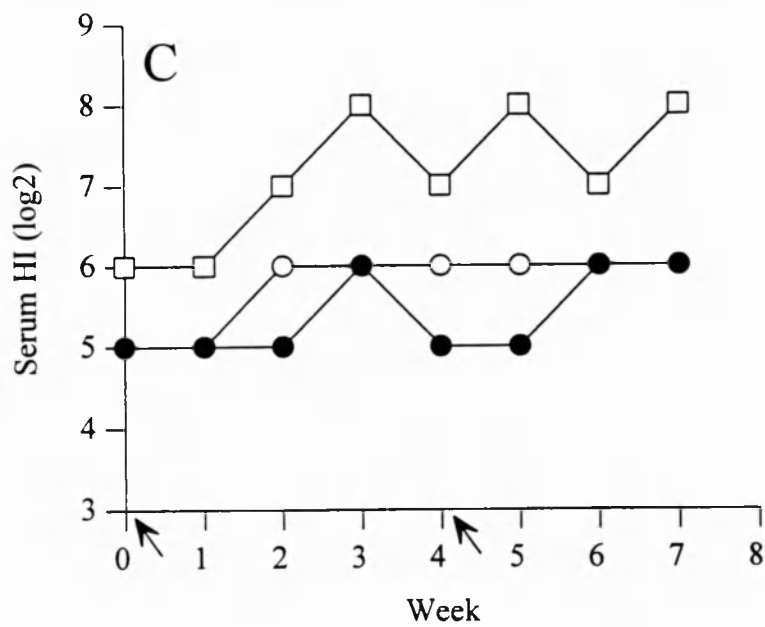
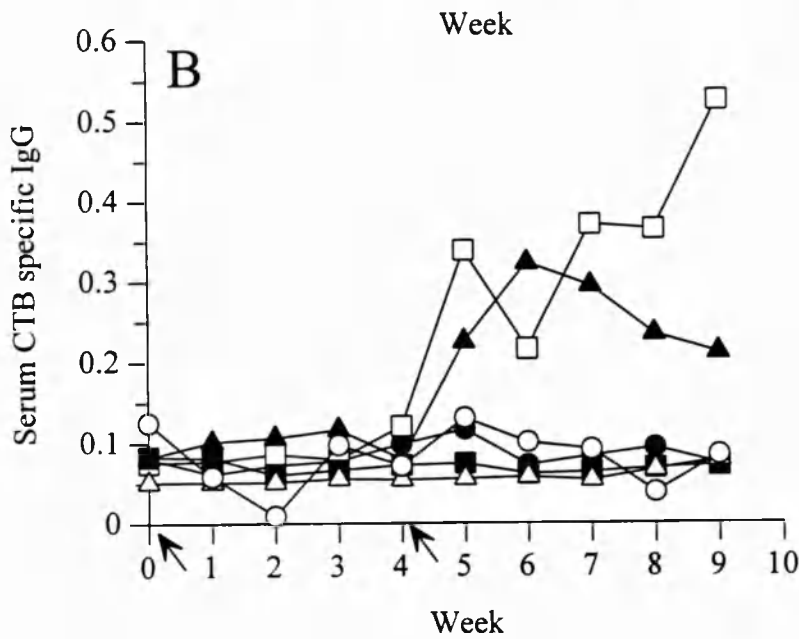
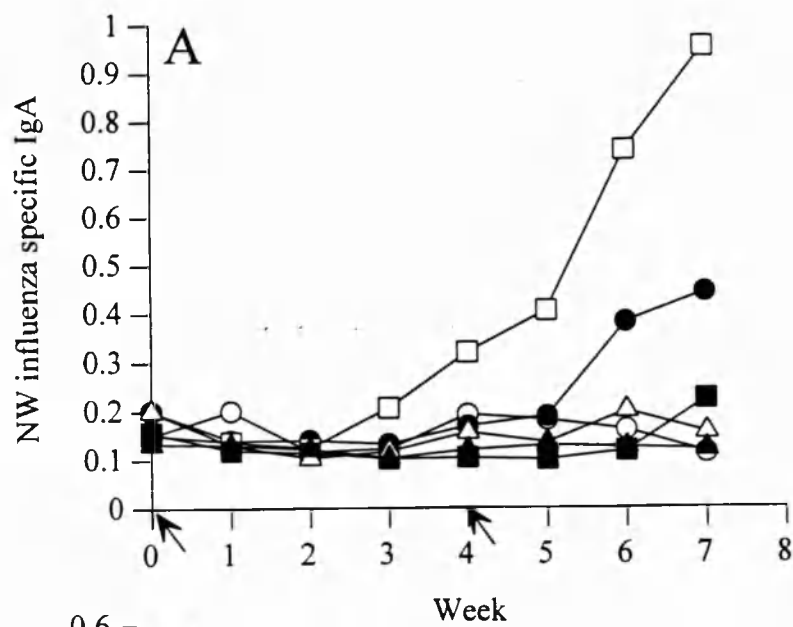
Intranasal vaccination with influenza virus and CTB induced influenza specific IgA in only 2 of the 6 ponies (figure 5.1.1, graph A), as detected by ELISA (see chapter 2.2.3). These ponies, D and C, were both previously infected and had received the CTB/S89 mixture and CTB/S89 SPDP respectively. The results are shown as raw ELISA absorbence values. No increases in influenza specific nasopharyngeal IgG were observed (data not shown).

Figure 5.1.1 Antibody responses in response to intranasal vaccination with different CTB/S89 formulations.

Figure 5.1 describes the immunoglobulin responses to two intranasal applications of the CTB/S89 based formulations (as described in 5.1), on week 0 and 4 (open arrows). Graph A, shows the changes in influenza specific IgA in nasal washes expressed as raw ELISA data (Ab.490nm). Graph B shows the changes in CTB specific IgG in serum (Ab.490nm). Graph C shows the change in serum haemagglutination inhibition (HI) titre in serum (log₂).

| | | | |
|---------------------|---------------|--------------|-------------|
| | S89/CTB Glut. | S89/CTB SPDP | S89/CTB mix |
| Naive | D70 | A58 | 26E |
| Previously infected | A | C | D |

○ A ● C □ D ■ D70 △ A58 ▲ 26E



5.1.3.2 *Systemic CTB and influenza specific antibody*

Serum CTB specific IgG, as measured by ELISA, was induced in two of the six ponies. These two ponies, D and 26E, both received the CTB/S89 mixture and showed an anamnestic response after the second booster application (figure 5.1.1, graph B). No serum CTB specific IgA was observed in any pony (data not shown).

Increases in influenza specific serum immunoglobulin, as measured by HI assay, were considered significant in only one pony, namely pony D. This pony had previously been infected with influenza and had received two applications of the CTB/S89 mixture (figure 5.1.1, graph C).

5.1.4 **Conclusions**

This set of experiments was concerned with identifying the optimum combination of CTB and influenza antigens. After examination of the data, by comparison of the magnitude of nasopharyngeal and systemic humoral responses, it was concluded that a mixture of the two components was superior to the CTB/S89 conjugation products.

The two conjugation procedures were selected from the literature as being suitable for conjugating influenza virus and CTB together. Glutaraldehyde is a well known cross-linking reagent, while SPDP because of its hetero-bifunctional nature was thought to cross-link the proteins in a more uniform controlled manner. Some preliminary studies (results not shown) were carried out to examine the conjugation of glutaraldehyde and SPDP, with a model antigen, bovine serum albumin (BSA). Each conjugate was shown to

retain its GM1 specific binding activity and antigenicity of both proteins as tested by ELISA (see table 5.1.2).

Table 5.1.2, GM1 binding capacity of BSA-CTB conjugate as conjugated with the hetero-bifunctional reagent SPDP.

| Revealing antibody | Antigen added to ELISA plate | | |
|--------------------|------------------------------|------|----------------------|
| | CTB | BSA | BSA/CTB Conjugate |
| α CTB | 0.7 | 0.04 | 0.65 |
| α BSA | 0.05 | 0.05 | 0.64 |

Footnotes:

The ELISA plate was coated with GM1 ganglioside, the three antigens (CTB, BSA, or the CTB/BSA conjugate) were added and allowed to bind to the GM1. After washing, the presence of antigen was revealed with either anti-CTB or anti-BSA antibodies. The table above shows the amounts of these antigens in ELISA absorbance values (Ab. 490nm).

Studies using 2D immuno-electrophoresis on these conjugates identified qualitative differences in the conjugates derived from the two methods tested. SPDP resulted in the generation of a uniform conjugate comprising BSA which was conjugated to one CTB chain. Glutaraldehyde conjugation, on the other hand, produced BSA conjugates of four distinct sizes, suggesting the inclusion of 1, 2, 3, or 4 CTB chains (gels not shown). Further ELISA studies using both GM1 and anti-BSA capture elements suggested that when either conjugation method was used, approximately 66% of the total amount of BSA

was successfully conjugated to CTB (data not shown). Given this suspicion that the S89/CTB conjugates may have contained free CTB, it is all the more surprising that the S89/CTB conjugates performed so poorly in the vaccination studies. Perhaps providing some indication that the conjugation conditions were sufficiently harsh to damage the antigenicity and adjuvanticity of the CTB.

Recent evidence has suggested that the immunogenicity of the CTB homologue, *E. Coli* heat-labile enterotoxin B subunit, may be a function of its GM1 binding activity (Nashar *et al.*, 1996). However, the adjuvanticity of CTB has been suggested to be a consequence of trace impurities of whole CT (Tamura *et al.*, 1994 and 1995). It has been shown that the glutaraldehyde treatment of CT decreased both the activity of cholera toxin A chain and the GM1 binding activity of the B chain (Liang *et al.*, 1989). Despite this, glutaraldehyde and SPDP conjugates of CTB and antigen have been used successfully by several authors (Bessen and Fischetti, 1988; Liang *et al.*, 1989).

However, it is interesting to note that in the experiments described here, the only two ponies to demonstrate a systemic CTB specific immune response, which was anamnestic after boosting, received the CTB/S89 mixture vaccine (figure 5.1.1, graph B). This is unlikely to be because of CTB dose as the SPDP-conjugated vaccines contained the highest dose of CTB, and therefore would further suggest that either the antigenicity or the adjuvanticity of the CTB was impaired during the conjugation process.

5.2 Intranasal vaccination of naïve ponies.

5.2.1 Introduction

Intranasal vaccination of ponies, which had been previously infected with influenza, induced influenza specific IgA in the nasopharynx (as described in 5.1). The 6 month old “naïve” group of animals, however, did not show any detectable mucosal antibody responses. It was decided that these three ponies, already twice intranasally vaccinated with a CTB/S89 based vaccine, would be boosted again this time with a simple mixture of CTB and influenza antigen. The animals were then experimentally infected and the clinical outcome to challenge investigated.

5.2.2 Methods

5.2.2.1 *Animals and vaccination protocols*

Three ponies, namely the previously naïve animals used in the experiment in 5.1 (A58, D70, and 26E), having received two applications of the different vaccine preparations were vaccinated intranasally for a third time. The third application of vaccine was given 35 weeks after the second intranasal vaccination, and was composed of a mixture of CTB (100µg) and heat inactivated A/Equi/2/Sussex/89 (300µg). The vaccinations were performed using the pasteur pipette method as described in 4.2.1. A fourth pony (pony 001) received two applications, 4 weeks apart, of CTB (100µg) alone and was used as a negative control. Ponies P16 and P18, were included in the challenge as further negative control animals and had received nothing at all.

5.2.2.2 Pony sampling and challenge

After vaccination, nasal wash and serum samples were taken periodically to monitor mucosal and systemic antibody responses. All ponies were experimentally infected by exposure to a nebulised aerosol of infectious virus (A/equi/2/Sussex/89), as described by Mumford *et al.* (1990), 3 weeks after the final intranasal boosting. After challenge, nasal wash and serum samples were taken to monitor antibody responses, heparinised blood samples were taken and the isolated PBMC (see 3.2.2) were used in influenza specific ELISPOT assays (see 3.2.3).

5.2.3 Results

5.2.3.1 ELISA antibody responses

Two of the 3 ponies showed a slight, transient nasopharyngeal influenza specific antibody response to the third intranasal application of CTB/S89 based vaccine (figure 5.2.1). None of the ponies showed serum influenza specific IgG responses, as measured by ELISA, before or after the third intranasal vaccination. All vaccinated ponies showed little or no influenza specific antibody, in nasal washes or serum, at the time of challenge. A fourth pony which received CTB alone showed no influenza specific antibody at any time after vaccination until the challenge (data not shown). After the experimental influenza infection all ponies showed marked increases in both serum and nasal wash influenza specific antibody (data expressed as arbitrary units AU, see chapter 2.5).

5.2.3.2 Nasal wash VN responses

Nasal wash virus neutralisation (VN) activities were assayed in tissue culture (see 2.2.10). The three vaccinates did not show any VN activity on day 0 (day of third vaccination) or day 21 (challenge -1). However, VN antibody could be detected by day 15 and was still detectable up to 42 days after infection. Negative control ponies (P16 and P18) showed equivalent levels of VN antibody after infection as that observed in the vaccinated animals 15 days post challenge.

Table 5.2.1, VN antibody measured in nasal washes before and after experimental infection (expressed as neutralisation indices, see 2.2.10).

| Pony | Vaccine | Day | | | |
|------|---------|-------|--------------|---------------|---------------|
| | | Day 0 | Challenge -1 | Challenge +15 | Challenge +42 |
| A58 | + | 0 | ND | 2.25 | ND |
| D70 | + | 0.4 | -0.125 | 0.625 | ND |
| 26E | + | ND | 0.325 | 1.5 | 1.5 |
| P16 | - | ND | ND | 2.375 | ND |
| P18 | - | ND | ND | 2.625 | ND |

5.2.3.3 Protection from challenge

All ponies were challenged 21 days after the third vaccination. Briefly, none of the three vaccinated ponies were resistant to infection and showed typical signs of elevated rectal temperatures from day 2-9 post infection, dry cough, and mucopurulent nasal discharge. The pony that received CTB alone, and the negative control ponies that received nothing, all became infected with clinical signs of similar magnitude and duration (see table 5.2.2).

Figure 5.2.1 Antibody responses to a third intranasal application of CTB/S89 based vaccine in naive ponies, and experimental infection.

Figure 5.2.1 shows the antibody responses in naive ponies vaccinated as described in 5.2. Day 0 corresponds to the final boosting vaccination prior to challenge, or week 35 in the vaccination schedule. Graph A shows the nasal wash influenza specific IgA and IgG (expressed as arbitrary units, as a function of total isotype), and IgG in serum (Ab.490nm), from pony A58. Graph B shows results from pony 26 E, and graph C from pony D70.

○ Nasal wash IgA ● Nasal wash IgG □ Serum IgG

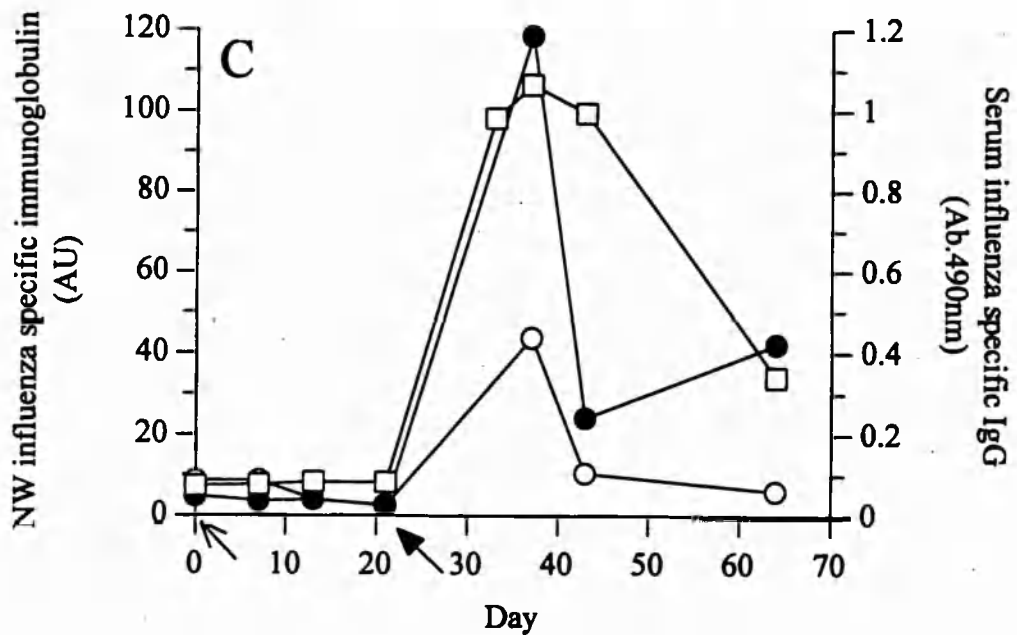
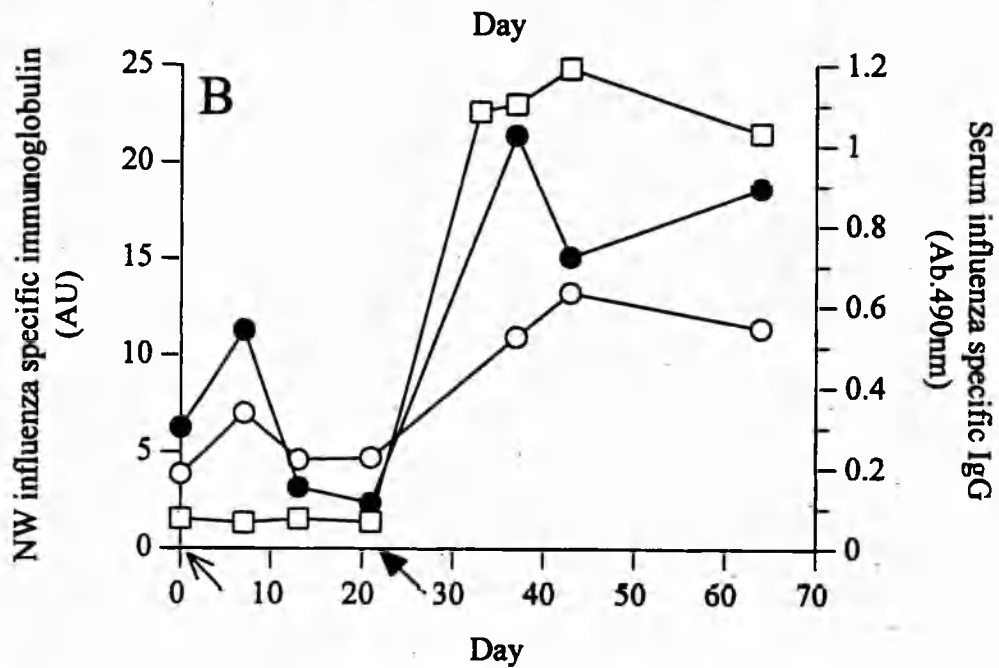
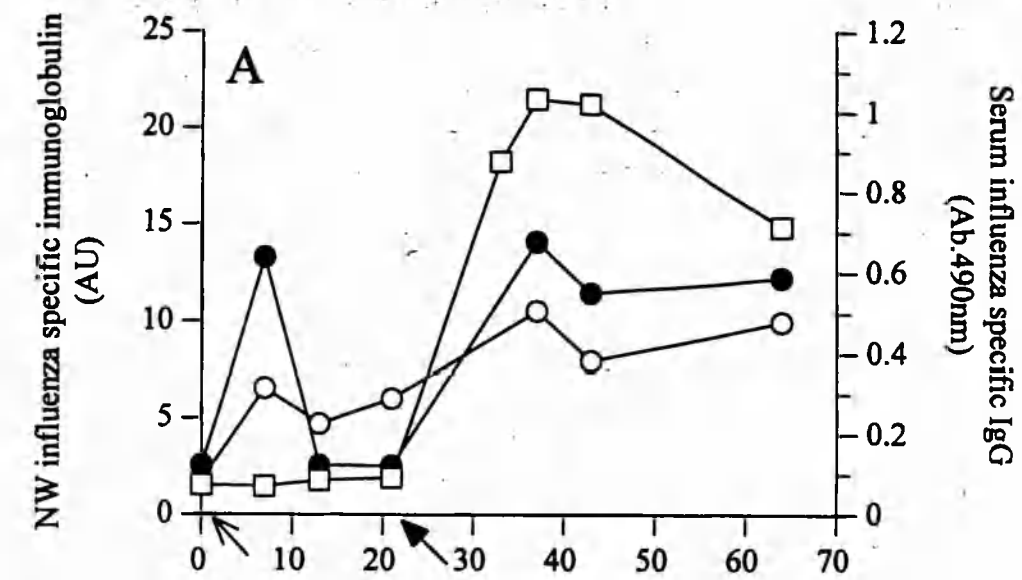


Table 5.2.2 Clinical outcome from experimental infection of intranasally vaccinated naive ponies.

| | CTB alone | | | A58 | | | D70 | | | 26E | | |
|-----|-----------|---|----|-----|---|---|-----|---|----|-----|---|----|
| Day | P | C | N | P | C | N | P | C | N | P | C | N |
| 0 | - | - | - | - | - | - | - | - | - | - | - | - |
| 1 | - | - | - | - | - | - | - | - | - | - | - | - |
| 2 | + | - | + | + | + | - | + | - | - | + | - | - |
| 3 | ++ | + | + | + | + | + | ++ | - | + | + | - | - |
| 4 | ++ | + | - | ++ | + | + | ++ | - | + | - | + | - |
| 5 | + | + | - | - | + | + | ++ | + | + | + | + | - |
| 6 | - | + | ++ | ++ | + | + | + | + | ++ | ++ | + | + |
| 7 | - | + | - | ++ | + | + | + | + | + | + | + | - |
| 8 | - | + | - | + | + | - | ++ | + | - | ++ | + | - |
| 9 | - | - | - | + | + | - | - | + | - | - | + | - |
| 10 | - | + | - | + | - | - | + | - | - | + | - | ++ |
| 11 | - | - | + | - | - | - | - | - | - | - | - | - |

Footnotes:

Day = Day post challenge.

P = Pyrexia.

C = Cough.

N = Muco-purulent nasal discharge.

+

++ = Moderate clinical sign observed.

5.2.3.4 *Influenza specific ASC responses after infection*

All ponies, vaccinates or controls, showed influenza specific antibody secreting cell responses (ASC) in the peripheral blood mononuclear cells (PBMC) after infection (figure 5.2.2). These cells were detected in PBMC from day 5, reaching a peak at around day 12, after infection. There was a markedly greater number of influenza specific IgG ASC on days 11 and 12 in the intranasally vaccinated ponies as compared with the unvaccinated controls, or the pony which had vaccinated with CTB alone. There was no obvious difference in the influenza specific IgA producing ASC responses.

The surprisingly high responses seen in pony P16 on day 11 after infection were unusual with respect to our previous experience with negative control ponies ($n \cong 10$).

Furthermore, on this day the "spots" were of an unusual nature in that they were very small, but still retained the diffuse edges, characteristic of ASC ELISPOTS.

Figure 5.2.2 Influenza specific ASC in the peripheral blood after an experimental infection.

Figure 5.2.2 shows a comparison of the ASC responses in the PBMC after an infection between a group of negative control ponies (001, P16 and P18) and a group intranasally vaccinated naive ponies (as described in 5.2, D70, A58, and 26E).

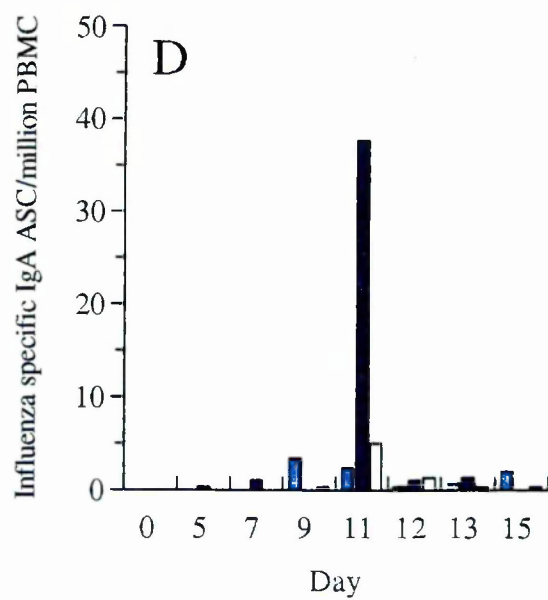
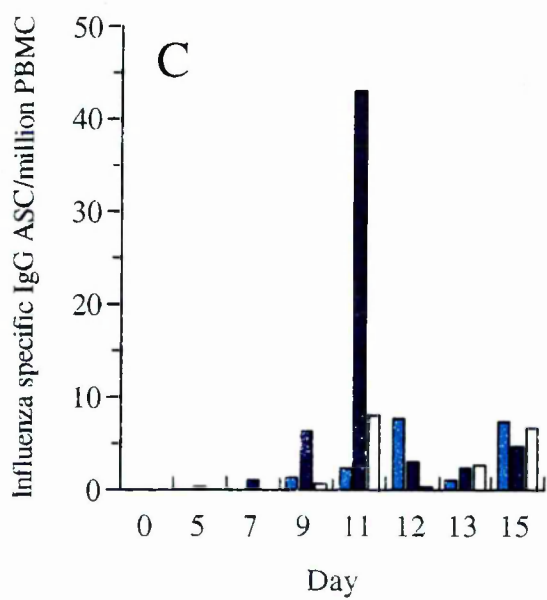
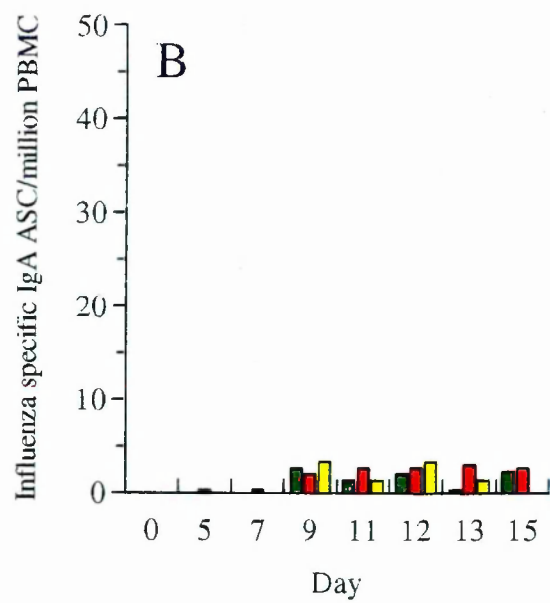
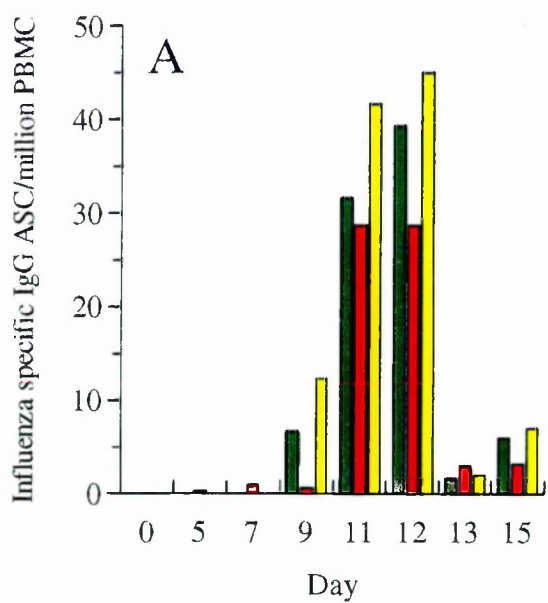
Graph A, influenza specific IgG ASC in the intranasally vaccinated ponies.

Graph B, influenza specific IgA ASC in the intranasally vaccinated ponies.

Graph C, influenza specific IgG ASC in the negative control ponies.

Graph D, influenza specific IgA ASC in the negative control ponies.

■ D70 ■ A58 □ 26E
■ 001 ■ P16 □ P18



5.3 Intranasal vaccination of systemically primed ponies.

5.3.1 Introduction

The experience with the naive ponies showed that i.n. vaccination with inactivated influenza antigens, did not elicit a protective immunological response. The experiment described in 5.1 demonstrated that prior exposure to influenza virus was a requisite for a successful response to mucosal vaccination with CTB/S89. The contribution of systemic vaccination to the induction of mucosal immune responses after mucosal boosting has been the subject of a number of conflicting studies, and probably depends on factors such as host, antigen, and the use of a carrier. Studies in mice have demonstrated a requirement for systemic priming before mucosal boosting (Barzu *et al.*, 1996), whilst others have demonstrated a preference for mucosal priming and boosting (Wu and Russell, 1994).

On the basis of these reports and the results of the studies described in 5.1 and 5.2, a third experiment was designed to investigate the role of systemic priming in the induction of mucosal immunological responses. A group of six yearlings, each without clinical and serological history of influenza infection were primed intramuscularly with conventional inactivated whole virus vaccine and then boosted intranasally. The immunological responses were monitored and the clinical outcome of an experimental infection was investigated.

5.3.2 Methods

5.3.2.1 Vaccination and sample protocols of the ponies

Ponies which were to be systemically primed prior to intranasal boosting were vaccinated intramuscularly with 45µg of h.i. influenza virus (5.1.2.1) with alhydrogel® adjuvant [Superfos Biosector a/s, Denmark]. Although the concentration of haemagglutinin was not calculated, it was assumed to comprise approximately 30% of the total protein (from a review of the literature). Four and 8 weeks after priming, the animals were boosted by intranasal vaccination with 300µg of h.i. influenza virus, 100µg of cholera toxin B chain [Sigma, cat. no. C9903], supplemented with 2µg of cholera toxin (whole molecule [Sigma, cat. no. C8052]). CT was added to the vaccine mixture because it has been shown to act synergistically as an adjuvant for nasal influenza vaccines (Tamura *et al.*, 1994). Intranasal vaccination (i.n.) was performed with the nasopharyngeal applicator as described in chapter 4.2.2. Briefly, ponies that were to receive the vaccine were sedated using 0.2 ml of Domosedan [Veterinary Drug Company] intravenously. The vaccine was instilled in a total volume of 1 ml. Ponies were kept in a loose box overnight and observed at 2, 6, and 24 hours after vaccination in case of any adverse reaction. The submandibular lymph nodes were palpated and rectal temperatures were taken at these time points.

Nasal wash samples (see chapter 2.2.1) were taken to monitor the appearance of mucosal immunoglobulins, and whole blood samples were collected by jugular venipuncture for measurement of circulating immunoglobulins and ELISPOT assays (for methods of PBMC isolation and ELISPOT assays see 3.2.1 and 3.2.2 respectively).

A further two ponies (804 and B40) were intramuscularly primed and received no further vaccinations. Serum samples were taken from these ponies and the HI antibody monitored.

The six intranasally vaccinated ponies were experimentally infected by exposure to infectious aerosol, as described by Mumford *et al.* (1990), 3 weeks after the final

intranasal boosting. Also included in the challenge were four negative control ponies with no clinical history of influenza infection or vaccination (V4, V7, V9, and V14), and two positive controls that had been experimentally infected six months previously (40A and A51). These animals had recovered completely from their previous infection and were clinically normal. Previous studies have shown that nasal shedding of virus after experimental infection lasts for approximately 7 days. The positive control ponies were expected to be immune from rechallenge on the basis of previous studies carried out in this department (Hannant *et al.*, 1988b).

5.3.2.2 *Nasopharyngeal swabs and sample manipulation*

Nasopharyngeal swabs were taken, put into virus transport medium (VTM, PBS containing 2% tryptose phosphate broth [ICN Flow, cat. no.16-821-49], 100 IU/ml penicillin [ICN Flow cat. no.16-702-49], 100 µg/ml streptomycin [ICN Flow, cat. no.16-702-49] and 5 µg/ml amphotericin B [Sigma, cat. no. A-2942] and placed on ice for transport to the laboratory. The swabs were squeezed using sterile forceps, the extracts clarified by centrifugation (1500×g, 5 mins), and aliquoted into sterile soda glass vials before freezing at -70°C.

5.3.2.3 *Virus isolation from nasopharyngeal swab extracts*

Nasopharyngeal swab extracts were thawed rapidly in a 37°C waterbath. 30µl of stock penicillin / streptomycin [10,000 IU/µg per ml, ICN Flow cat. no.16-702-49, ICN Flow, cat. no.16-702-49 respectively] was added to each 1 ml of extract to raise the antibiotic level to around 400 units/µg per ml. Dilutions of the extracts were prepared ranging from

10^0 to 10^{-3} in \log_{10} steps, in PBS containing 2% tryptose phosphate broth [ICN Flow, cat. no. 16-821-49] and 500 units/ $\mu\text{g ml}^{-1}$ penicillin and streptomycin [ICN Flow cat. no. 16-702-49, ICN Flow, cat. no. 16-702-49]. Nasal swab extracts were kept on wet ice until use. 100 μl volumes of the titrated extracts were injected, in duplicate, into the allantoic cavity of 10 day old fertile hens' eggs. The eggs were incubated for 72 hours at 34°C , and then placed at 4°C overnight. Allantoic fluid was harvested from each individual egg and dilutions (neat, 1:2, and 1:4), of each harvested sample were prepared in V bottomed 96-well plates (in 25 μl volumes). 25 μl volumes of 1% chicken red blood cells were added to all wells and after 30 mins the plates were read for haemagglutination. The EID_{50} per ml of each swab extract was calculated using the Karber formula (Dougherty, 1964).

5.2.2.4 *Detection of influenza virus nucleoprotein in the nasal swab extracts*

Nucleoprotein (NP) of influenza virus was detected by a variation of the immunocapture ELISA as described by Cook *et al.* (1988). Briefly, NP present in the nasal swab extract was captured by polyclonal anti-equine influenza (H3N8) bound to the wells of a 96 well ELISA plate. The bound NP was then revealed using an anti NP monoclonal antibody labelled with horseradish peroxidase.

5.3.3 **Results**

5.3.3.1 *HI antibody responses*

HI antibody was detected in the serum of all 6 of the vaccinated ponies after intramuscular vaccination. The intranasal boosting with CTB/S89 of primed ponies induced further increases in 5 of the 6 ponies (figures 5.3.1 to 5.3.6).

Intramuscular priming of ponies 804 and B40 induced transient but low amounts of serum HI antibody (figure 5.3.7). Log₂ HI titre of 804 never increased above 3 and the maximum titre for pony B40 (5) was seen 54 days after priming. Ponies 804 and B40 did not progress to challenge for two reasons. Firstly, they did not become available for this experiment until the intranasal boosting procedures were well underway, and so it was not possible to examine their responses in parallel. Secondly, it was decided more important to measure the induction and decline of primary vaccination induced antibody in these ponies because there is sufficient published and laboratory data to show that ponies with HI titres of less than 6 are susceptible to infection (Hannant *et al.*, 1988a, 1988b, 1989a, 1989b; Mumford *et al.*, 1983, 1988, 1990).

5.3.3.2 *Virus neutralising antibody responses*

The VN activity of nasal wash samples was investigated in tissue culture. At the time of challenge 4 of the 6 vaccinated ponies showed VN activity greater than those of the unvaccinated control animals 14 days after infection (figures 5.3.1 to 5.3.6).

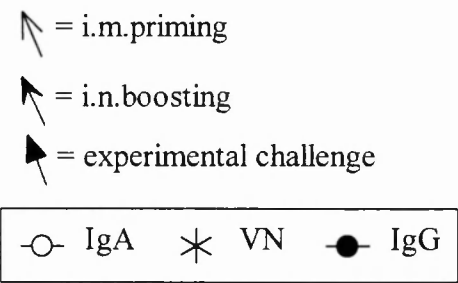
5.3.3.3 *ELISPOT responses*

After the first and second intranasal boosting vaccinations, significant numbers (≥ 20 ASC per 10^6 PBMC, *Nb*, on day 0 no ASC were observed, the figure 20 was considered an arbitrary figure of significance) of IgA and IgG ASC were detected in 5 of the 6 ponies. The ASC appeared in PBMC as early as 3-4 days after boosting, and peaked at day 5. The ASC responses following the second boost were not as great as that those seen after the first boosting vaccination (figures 5.3.1 to 5.3.6). The appearance of ASC coincided with

Figure 5.3.1 **Immunological responses of systemically primed ponies to intranasal boosting with CTB/influenza vaccine, and experimental infection.**

Data derived from a single pony : Pony F79

The nasopharyngeal and systemic antibody responses to intramuscular priming (day -28), intranasal boosting vaccination with S89 and CTB (2%CT) on days 0 and 21, and subsequent challenge with influenza virus (day 48).



Panel A, Influenza specific nasopharyngeal IgA (\bigcirc) and IgG (\bullet) expressed as arbitrary units (AU), as a function of total isotype, in relation to circulating influenza specific ASC, illustrated as a histogram, showing numbers of ASC /10⁶ PBMC.



Panel B, Influenza specific nasopharyngeal IgA (\bigcirc) and IgG (\bullet) expressed as raw ELISA data, and virus neutralisation activity (assayed on day 0, 46, and 62) of the nasal washes (expressed as a neutralisation index (NI), asterisk sign).

Panel C, Serum IgA (\bigcirc) and IgG (\bullet) (expressed as raw ELISA data, Ab.490nm) and serum haemagglutination activity (expressed as histogram).

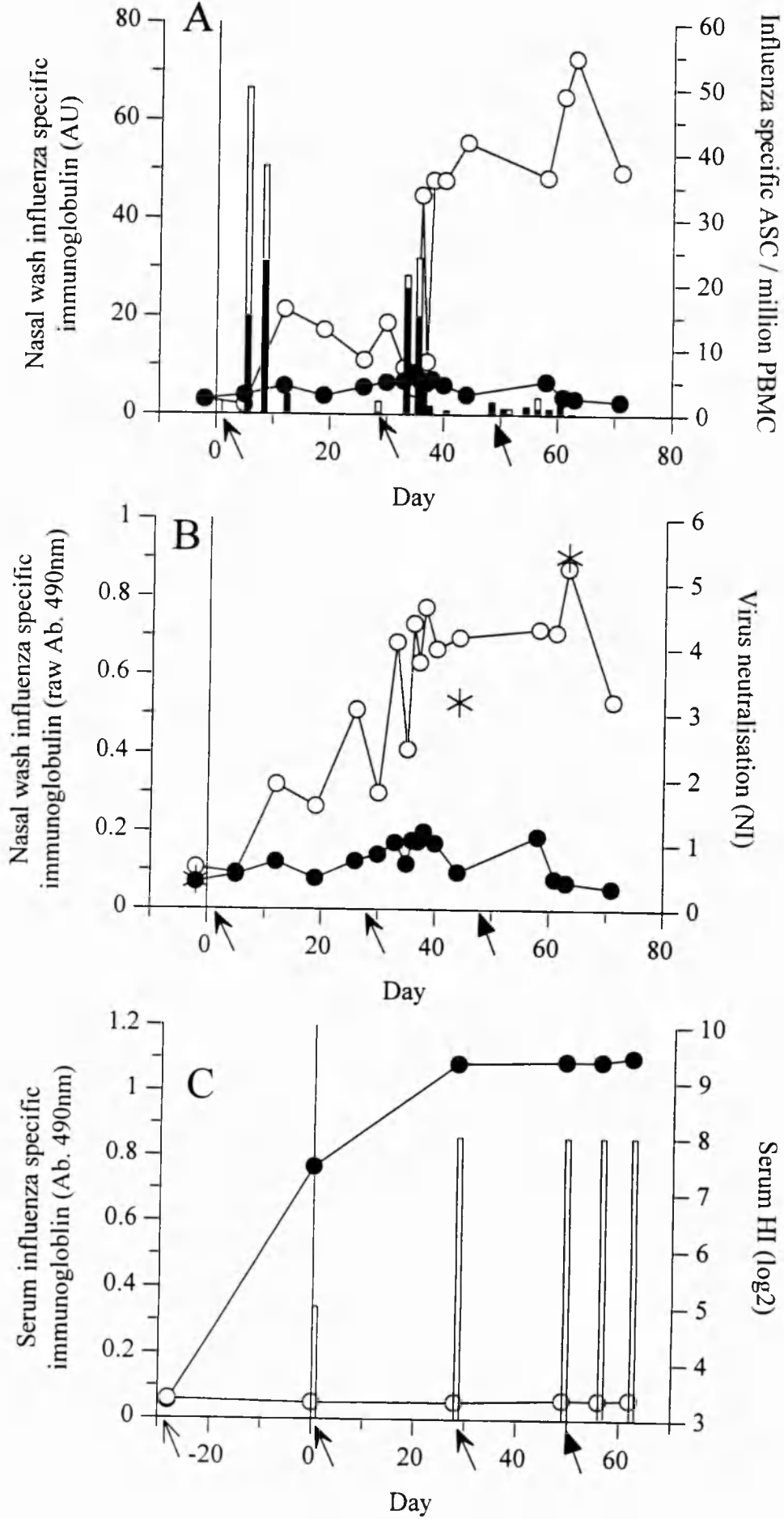
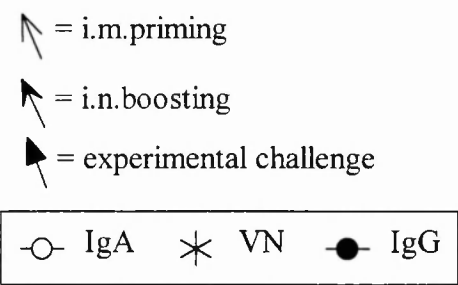


Figure 5.3.2 Immunological responses of systemically primed ponies to intranasal boosting with CTB/influenza vaccine, and experimental infection.

Data derived from a single pony: Pony 90F

The nasopharyngeal and systemic antibody responses to intramuscular priming (day -28), intranasal boosting vaccination with S89 and CTB (2%CT) on days 0 and 21, and subsequent challenge with influenza virus (day 48).



Panel A, Influenza specific nasopharyngeal IgA (○)and IgG (●) expressed as arbitrary units (AU), as a function of total isotype, in relation to circulating influenza specific ASC, illustrated as a histogram, showing numbers of ASC /10⁶ PBMC.



Panel B, Influenza specific nasopharyngeal IgA (○) and IgG (●) expressed as raw ELISA data, and virus neutralisation activity (assayed on day 0, 46, and 62) of the nasal washes (expressed as a neutralisation index (NI), asterisk sign).

Panel C, Serum IgA (○) and IgG (●) (expressed as raw ELISA data, Ab.490nm) and serum haemagglutination activity (expressed as histogram).

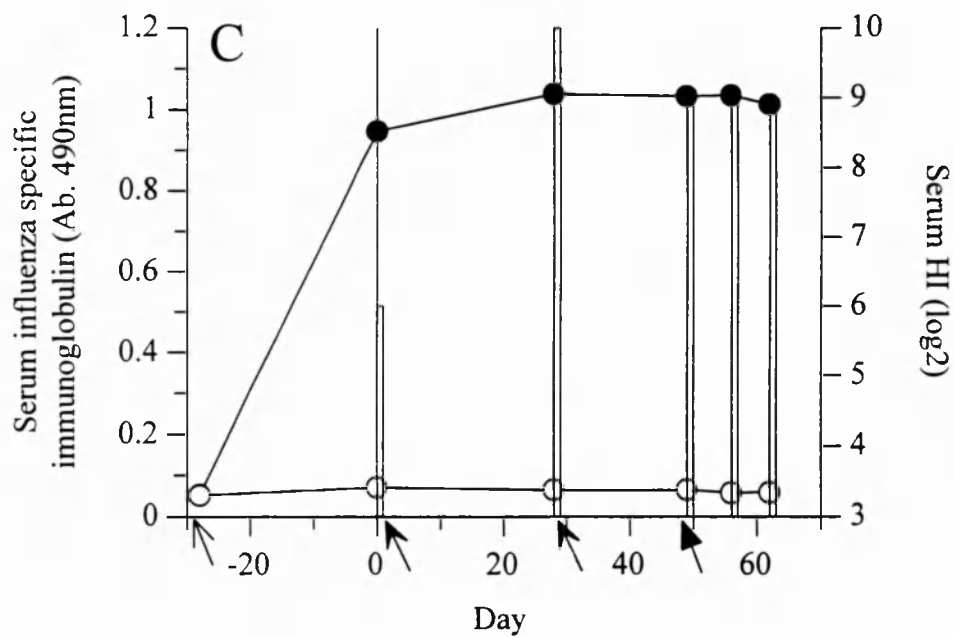
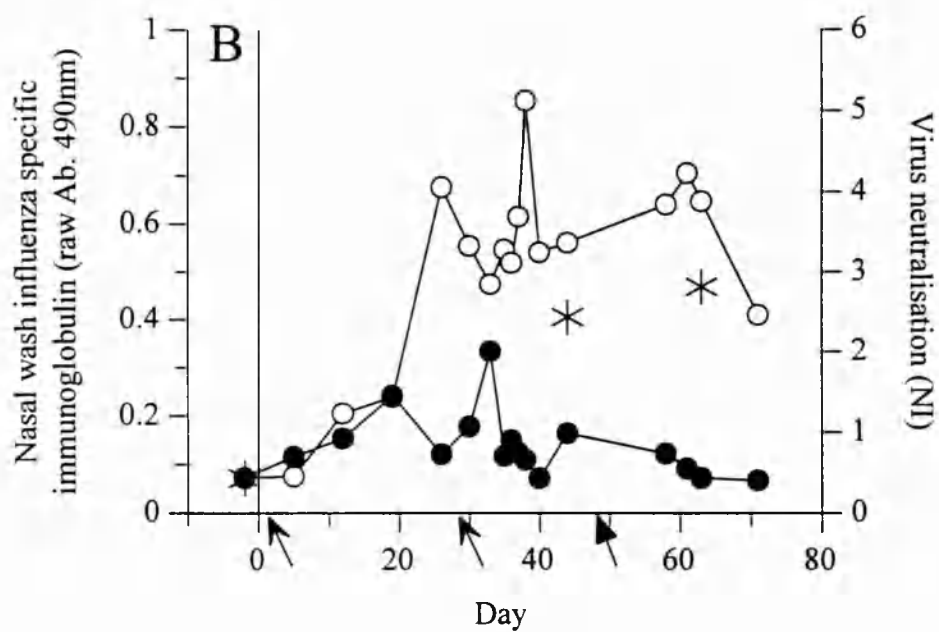
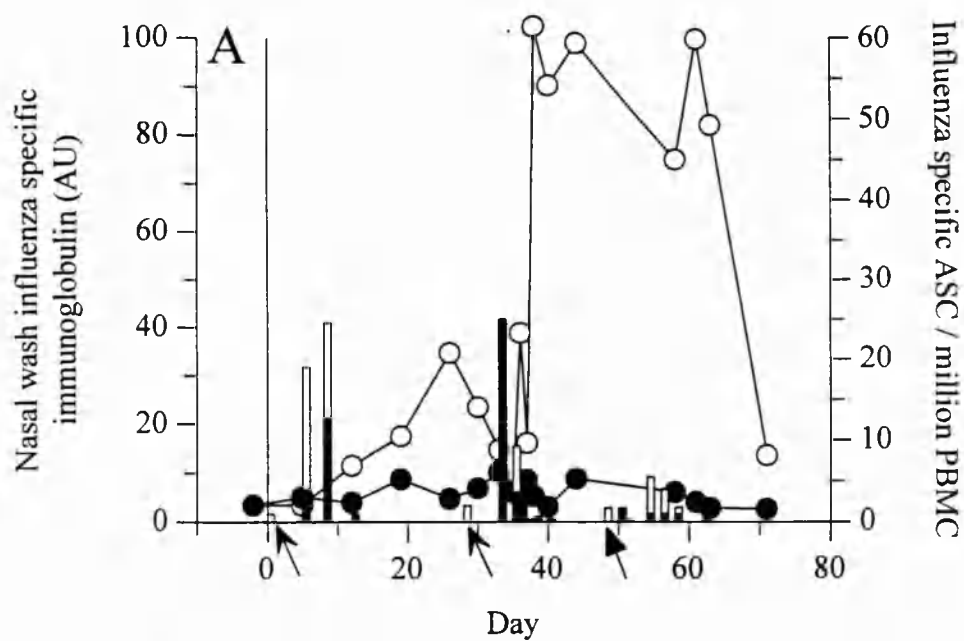
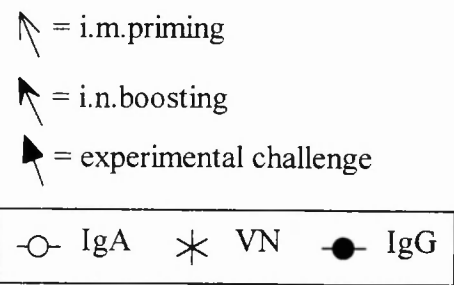


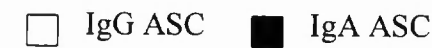
Figure 5.3.3 **Immunological responses of systemically primed ponies to intranasal boosting with CTB/influenza vaccine, and experimental infection.**

Data derived from a single pony: Pony 6431

The nasopharyngeal and systemic antibody responses to intramuscular priming (day -28), intranasal boosting vaccination with S89 and CTB (2%CT) on days 0 and 21, and subsequent challenge with influenza virus (day 48).



Panel A, Influenza specific nasopharyngeal IgA (○)and IgG (●) expressed as arbitrary units (AU), as a function of total isotype, in relation to circulating influenza specific ASC, illustrated as a histogram, showing numbers of ASC /10⁶ PBMC.



Panel B, Influenza specific nasopharyngeal IgA (○) and IgG (●) expressed as raw ELISA data, and virus neutralisation activity (assayed on day 0, 46, and 62) of the nasal washes (expressed as a neutralisation index (NI), asterisk sign).

Panel C, Serum IgA (○) and IgG (●) (expressed as raw ELISA data, Ab.490nm) and serum haemagglutination activity (expressed as histogram).

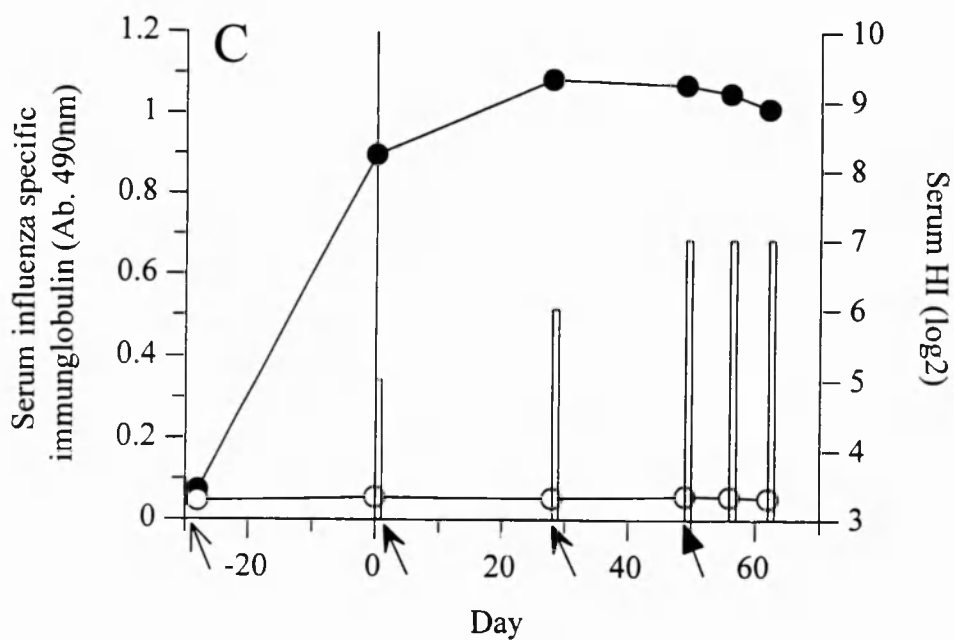
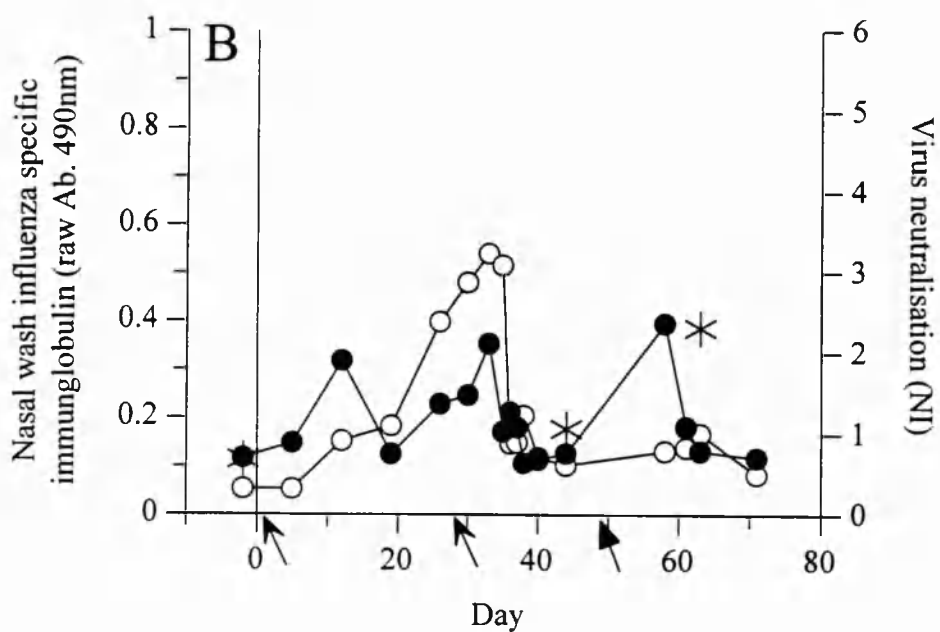
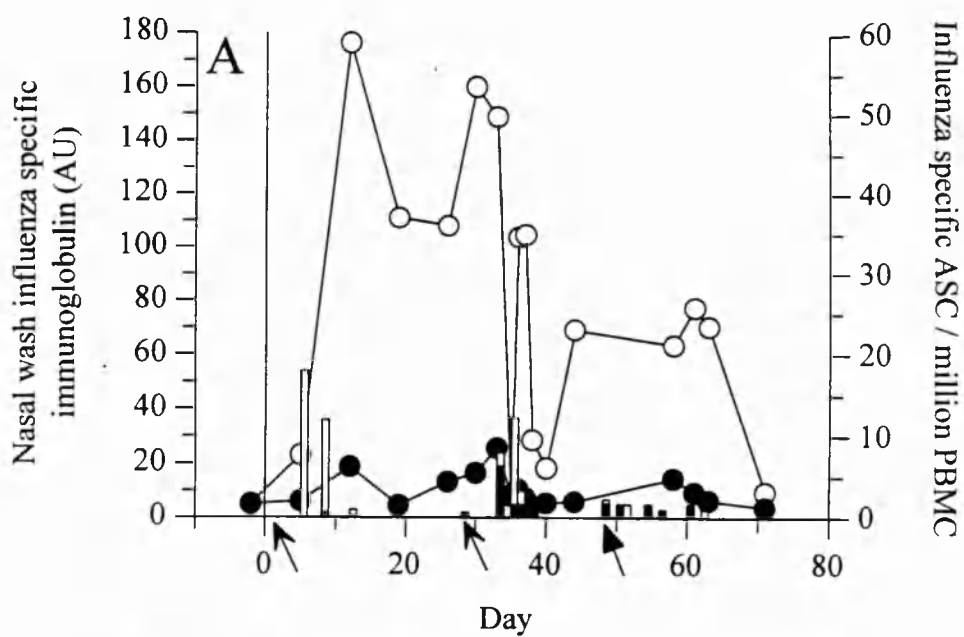
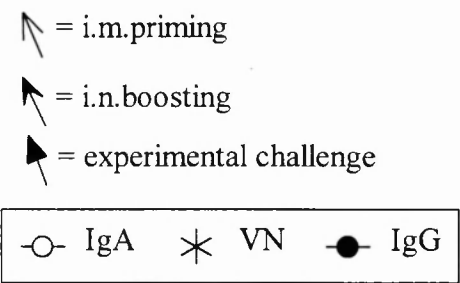


Figure 5.3.4 Immunological responses of systemically primed ponies to intranasal boosting with CTB/influenza vaccine, and experimental infection.

Data derived from a single pony: Pony 1669

The nasopharyngeal and systemic antibody responses to intramuscular priming (day -28), intranasal boosting vaccination with S89 and CTB (2%CT) on days 0 and 21, and subsequent challenge with influenza virus (day 48).



Panel A, Influenza specific nasopharyngeal IgA (\bigcirc) and IgG (\bullet) expressed as arbitrary units (AU), as a function of total isotype, in relation to circulating influenza specific ASC, illustrated as a histogram, showing numbers of ASC / 10^6 PBMC.



Panel B, Influenza specific nasopharyngeal IgA (\bigcirc) and IgG (\bullet) expressed as raw ELISA data, and virus neutralisation activity (assayed on day 0, 46, and 62) of the nasal washes (expressed as a neutralisation index (NI), asterisk sign).

Panel C, Serum IgA (\bigcirc) and IgG (\bullet) (expressed as raw ELISA data, Ab.490nm) and serum haemagglutination activity (expressed as histogram).

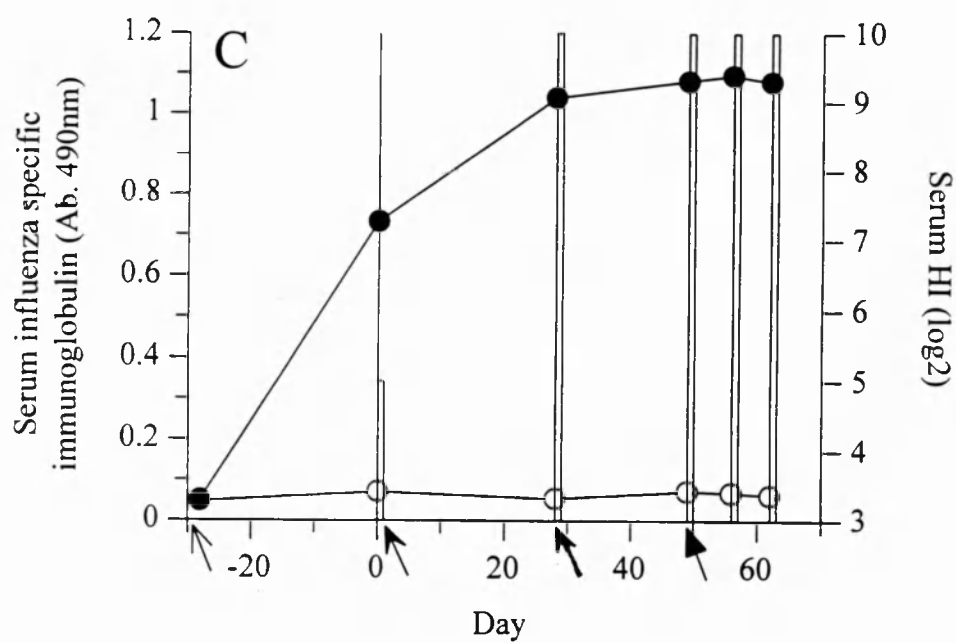
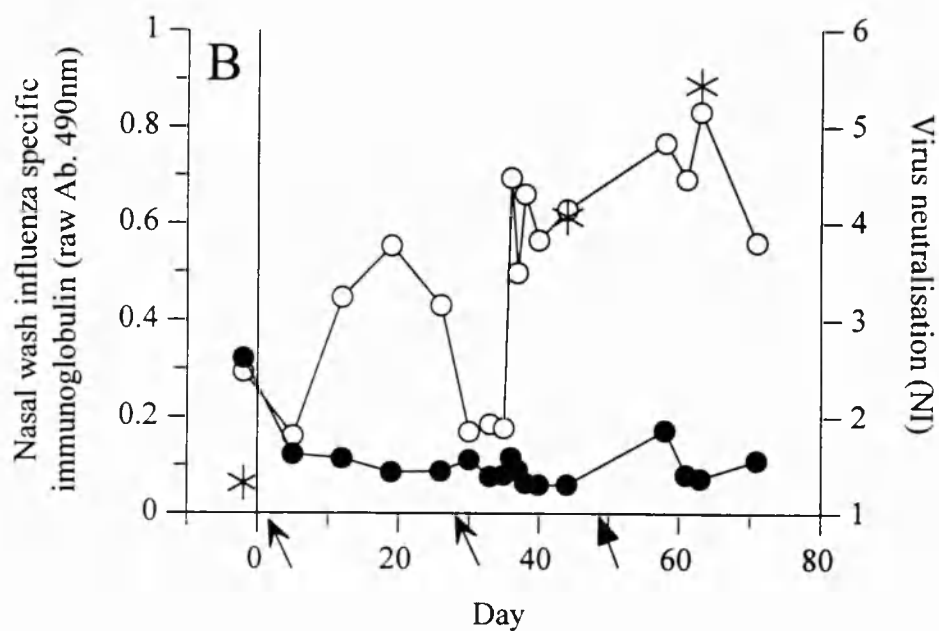
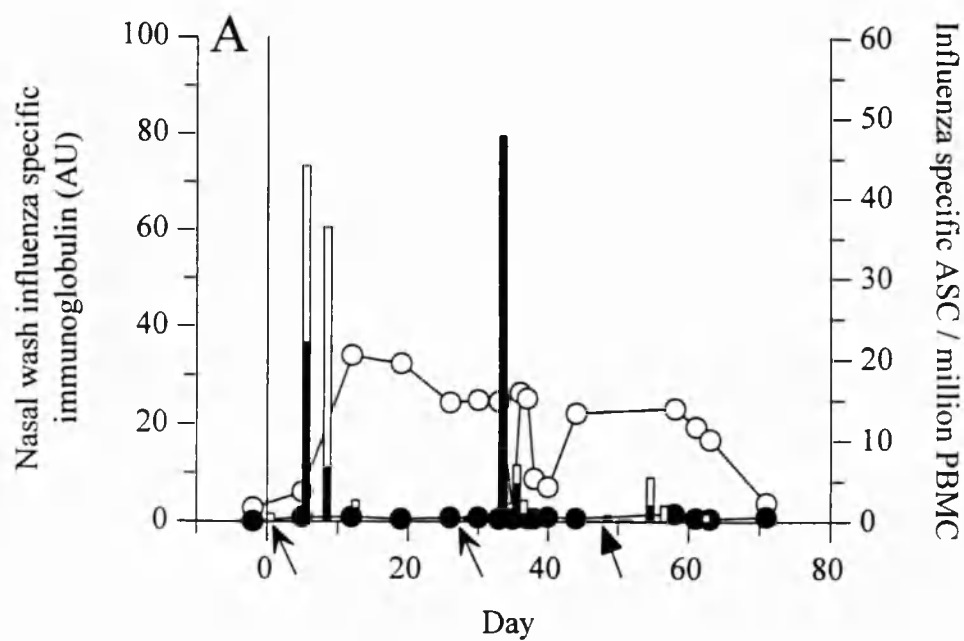
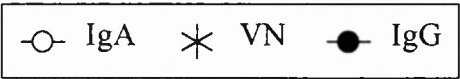


Figure 5.3.5 **Immunological responses of systemically primed ponies to intranasal boosting with CTB/influenza vaccine, and experimental infection.**

Data derived from a single pony: Pony O4B

The nasopharyngeal and systemic antibody responses to intramuscular priming (day -28), intranasal boosting vaccination with S89 and CTB (2%CT) on days 0 and 21, and subsequent challenge with influenza virus (day 48).

↖ = i.m.priming
↗ = i.n.boosting
➡ = experimental challenge



Panel A, Influenza specific nasopharyngeal IgA (○)and IgG (●) expressed as arbitrary units (AU), as a function of total isotype, in relation to circulating influenza specific ASC, illustrated as a histogram, showing numbers of ASC /10⁶ PBMC.

□ IgG ASC ■ IgA ASC

Panel B, Influenza specific nasopharyngeal IgA (○) and IgG (●) expressed as raw ELISA data, and virus neutralisation activity (assayed on day 0, 46, and 62) of the nasal washes (expressed as a neutralisation index (NI), asterisk sign).

Panel C, Serum IgA (○) and IgG (●) (expressed as raw ELISA data, Ab.490nm) and serum haemagglutination activity (expressed as histogram).

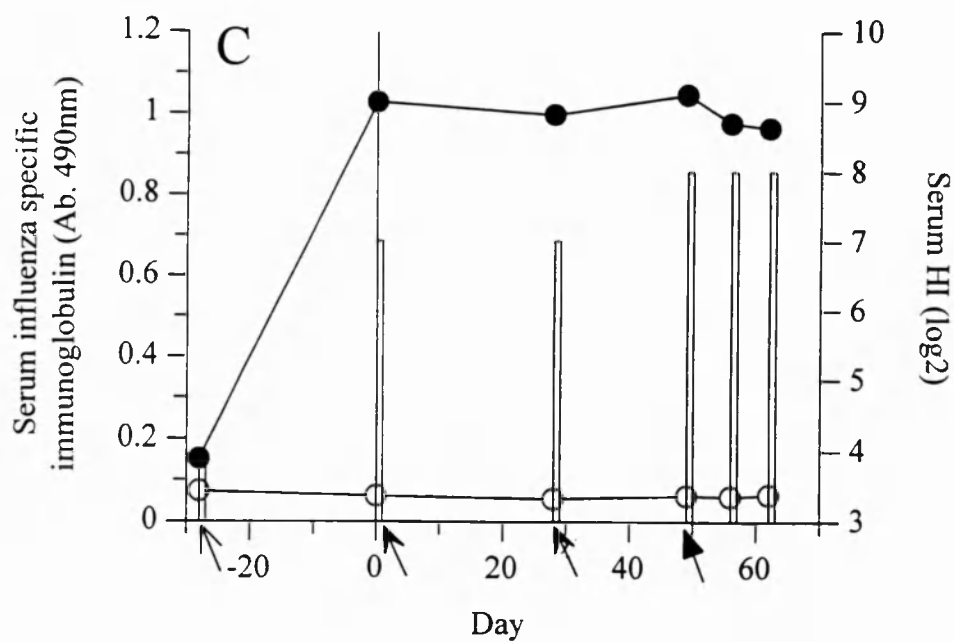
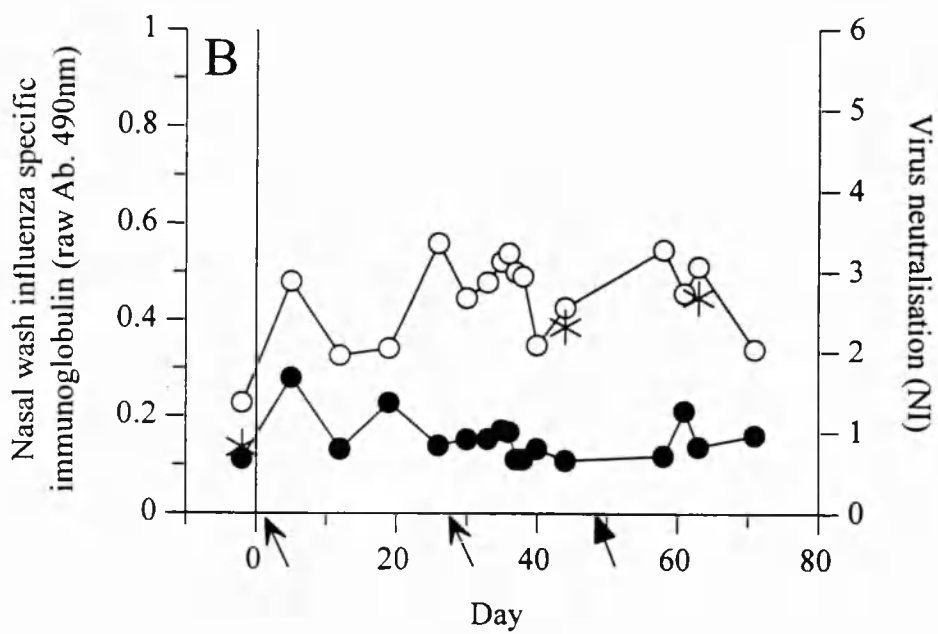
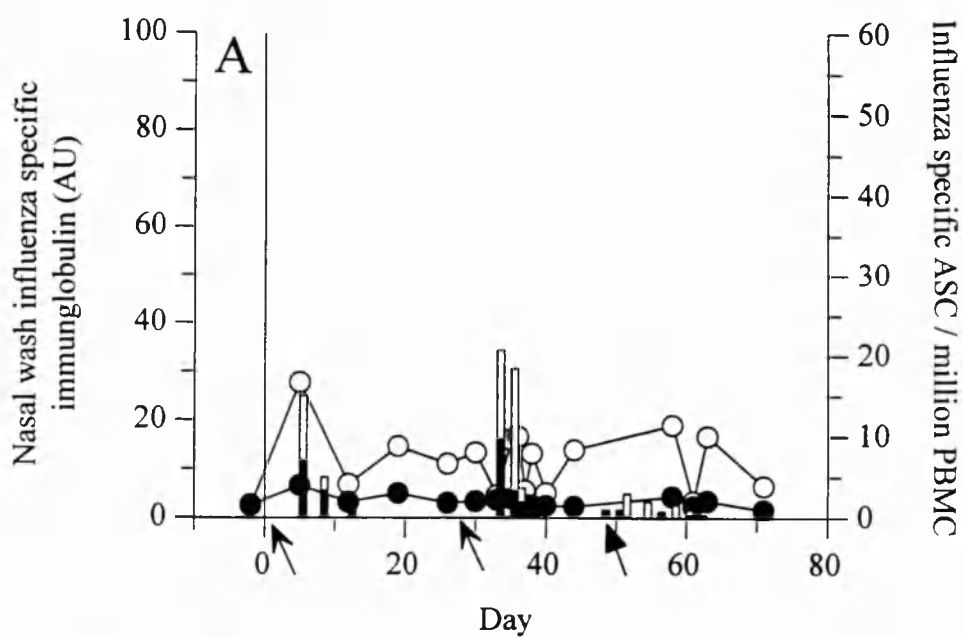
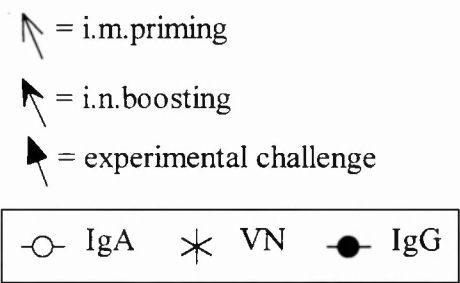


Figure 5.3.6 Immunological responses of systemically primed ponies to intranasal boosting with CTB/influenza vaccine, and experimental infection.

Data derived from a single pony: Pony C4D

The nasopharyngeal and systemic antibody responses to intramuscular priming (day -28), intranasal boosting vaccination with S89 and CTB (2%CT) on days 0 and 21, and subsequent challenge with influenza virus (day 48).



Panel A, Influenza specific nasopharyngeal IgA (○)and IgG (●) expressed as arbitrary units (AU), as a function of total isotype, in relation to circulating influenza specific ASC, illustrated as a histogram, showing numbers of ASC /10⁶ PBMC.



Panel B, Influenza specific nasopharyngeal IgA (○) and IgG (●) expressed as raw ELISA data, and virus neutralisation activity (assayed on day 0, 46, and 62) of the nasal washes (expressed as a neutralisation index (NI), asterisk sign).

Panel C, Serum IgA (○) and IgG (●) (expressed as raw ELISA data, Ab.490nm) and serum haemagglutination activity (expressed as histogram).

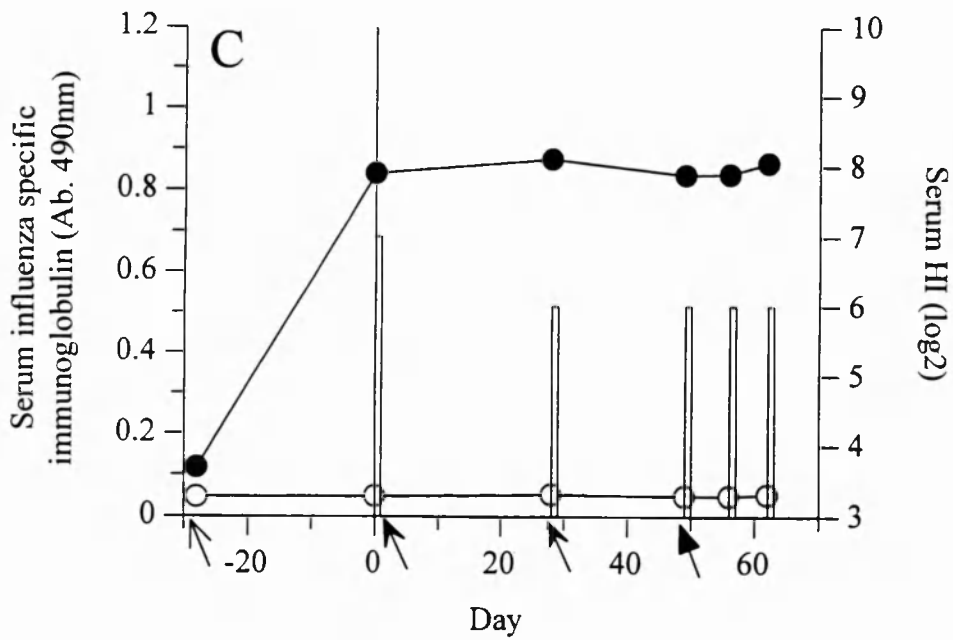
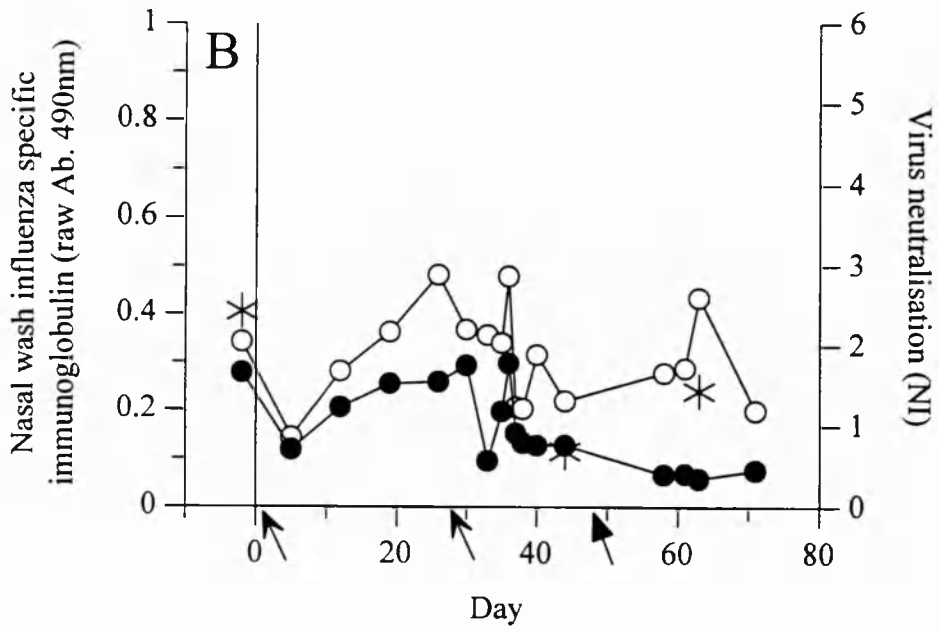
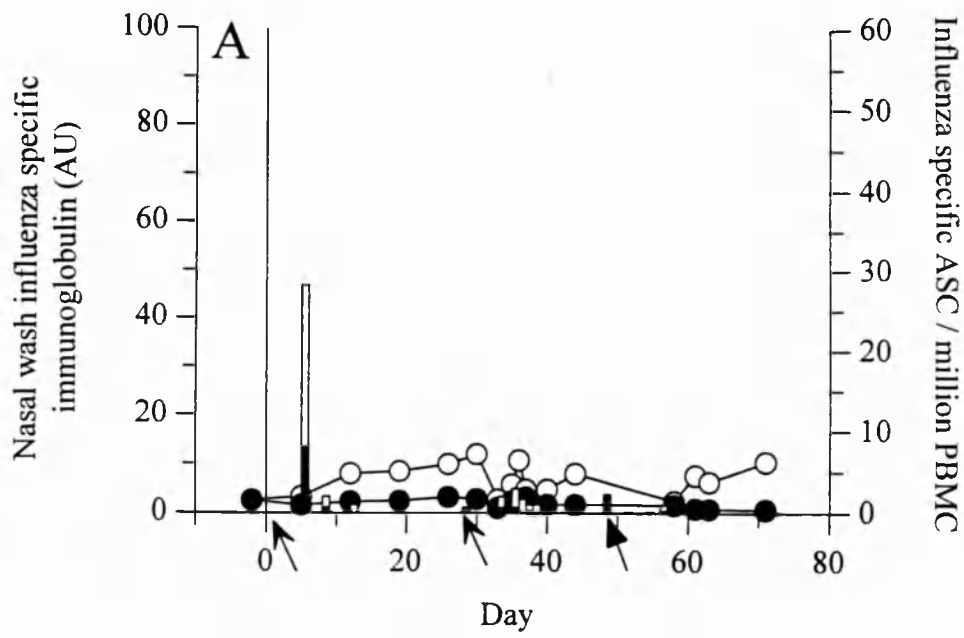
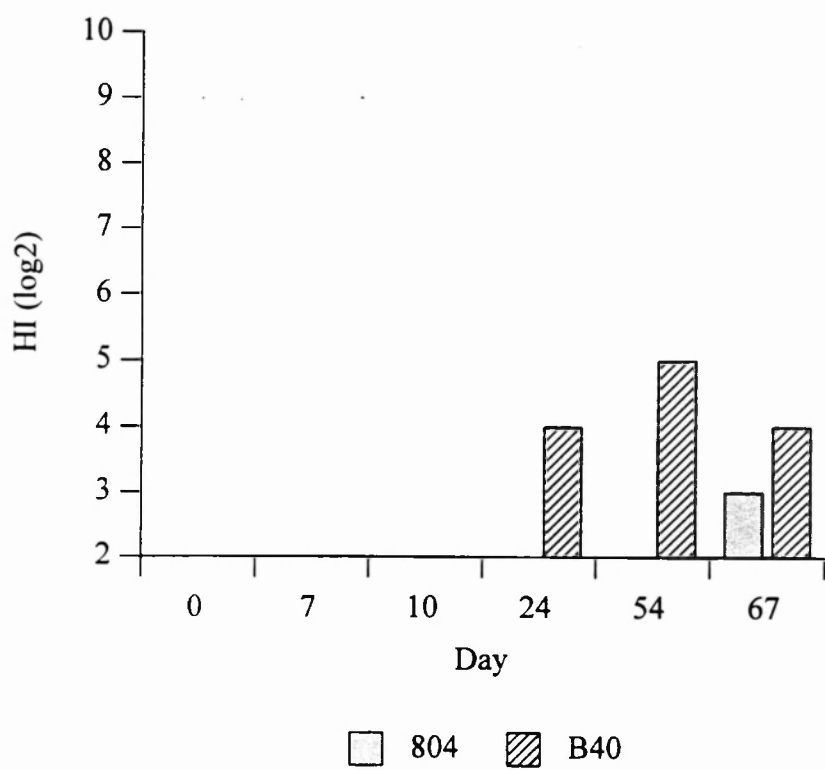


Figure 5.3.7 Serum haemagglutination inhibition (HI) titres of intramuscularly primed ponies.

Figure 5.3.7 shows that serum HI titres in two control ponies at days 0, 7, 10, 24, 54, and 67 after intramuscular priming with 45µg heat inactivated whole virus in Alhydrogel® adjuvant. The sensitivity of the HI assay with 4HAU per well is such that titres of less than 3 (\log_2) cannot be registered. Therefore, a value of 2 (\log_2) should be taken to mean $<1/8$ including 0.



or preceded the rise in influenza specific IgA (expressed in arbitrary units AU, see chapter 2.5) in the nasal secretions in 4 of those 5 ponies (figures 5.3.1 to 5.3.6).

5.3.3.4 Antibody responses as measured by ELISA

Increases in influenza specific IgA were detected in the nasal washes of all 6 of the vaccinated ponies. However, no increases in nasal wash influenza specific IgG were detected. The virus neutralisation activity (NI) of the nasal washes closely mirrored the levels of influenza specific IgA (raw absorbance data) present in the nasal wash (figures 5.3.1 to 5.3.6; graph B). Serum influenza specific IgG was detected in all 6 of the vaccinates after the intramuscular priming, this was further boosted by the intranasal vaccinations in four of the six.

5.3.3.5 Response to challenge infection

Unvaccinated control ponies

All 4 of the unvaccinated control ponies showed classical clinical signs of influenza infection after challenge (pyrexia, dry non-productive cough, ocular discharge, and mucopurulent nasal discharge, table 5.3.1).

Influenza specific ASC responses were detected in all 4 ponies, the kinetics of which seemed to be slower than those observed in the vaccinated pony group (figures 5.3.8 to 5.3.11). Increases in nasal wash and serum influenza specific antibody were also observed in all ponies (figures 5.3.8 to 5.3.11). Also NP was detected in all ponies in nasopharyngeal swab

Table 5.3.1 **Clinical signs of the unvaccinated control ponies.**

Figure 5.3.1 shows data derived from the experimental infection described in 5.3. The unvaccinated control ponies were scored for rectal temperatures (°F), cough, condition, ocular discharge, and nasal discharge. A "+" sign designates the severity of the clinical sign.

| Day | Pony number | | | | | | | | | | | | | | | |
|------|-------------|-------|------|-------------|----------------|-------|-------|------|-------------|----------------|-------|-------|------|-------------|----------------|-------|
| | V4 | | | | | | | | V7 | | | | | | | |
| | Temp | Cough | Cond | Oc Disch | Nasal Disch | Temp | Cough | Cond | Oc Disch | Nasal Disch | Temp | Cough | Cond | Oc Disch | Nasal Disch | Temp |
| C-2 | 99.8 | - | - | - | - | 101.5 | - | - | - | - | 100.6 | - | - | - | - | 101.5 |
| C-1 | 100 | - | - | - | - | 101.2 | - | - | - | - | 100.8 | - | - | + | - | 100.2 |
| C | 99.8 | - | - | + | - | 100.3 | - | - | - | - | 100.3 | - | - | + | - | 100.1 |
| C+1 | 99.6 | - | - | + | - | 100.7 | - | - | - | - | 101 | - | - | - | - | 100.4 |
| C+2 | 100 | - | - | + | + | 102.2 | - | - | + | - | 100.4 | + | - | + | - | 102.2 |
| C+3 | 100.2 | + | - | + | + | 102.6 | ++ | - | + | - | 101.6 | - | - | + | - | 103.6 |
| C+4 | 100.2 | + | - | + | + | 103.6 | + | - | - | + | 101.3 | - | - | + | + | 103.6 |
| C+5 | 101.2 | ++ | - | + | + | 103.4 | ++ | - | - | ++ | 102.4 | +++ | - | + | + | 102.2 |
| C+6 | 101.4 | ++ | - | + | ++ | 100.9 | ++ | - | + | ++ | 100.1 | ++ | - | + | ++ | 102.6 |
| C+7 | 99.6 | +++ | - | + | + | 101.4 | +++ | + | + | ++ | 100.6 | +++ | - | + | ++ | 102.4 |
| C+8 | 99.6 | + | - | + | ++ | 103.2 | +++ | - | + | ++ | 99.9 | ++ | - | + | + | 102.1 |
| C+9 | 100.1 | ++ | - | + | + | 99.8 | ++ | + | + | ++ | 99 | + | - | + | + | 101.1 |
| C+10 | 101.8 | + | - | ++ | + | 101.8 | +++ | + | - | ++ | 100.4 | + | - | + | ++ | 103.6 |
| C+11 | 99 | + | - | + | - | 101.4 | +++ | - | + | ++ | 99.8 | ++ | - | + | ++ | 101.1 |
| C+12 | 99.6 | + | - | + | + | 98 | ++ | - | + | + | 99.4 | ++ | - | + | ++ | 99 |
| C+13 | 99 | + | - | + | + | 100.6 | ++ | - | + | + | 99.8 | + | - | + | + | 101.8 |
| C+14 | 102.3 | - | - | + | + | 100 | ++ | - | - | + | 101.2 | - | - | + | + | 100.5 |

Table 5.3.2 Clinical signs of the vaccinated and previously infected ponies.

Figure 5.3.3 shows data derived from the experimental infection described in 5.3. The vaccinated (F79, 90F, 6431, 1669, 04B, C4D) and previously infected control ponies (A51 and 40A) were scored for rectal temperatures (°F), cough, condition, ocular discharge, and nasal discharge. A "+" sign designates the severity of the clinical sign.

| Day | Pony number | | | | | | | | | | | | | | | | | | | |
|------|-------------|-------|------|----------|-------------|-------|-------|------|----------|-------------|-------|-------|------|----------|-------------|-------|-------|------|----------|-------------|
| | F79 | | | | | 90F | | | | | 1669 | | | | | 6431 | | | | |
| | Temp | Cough | Cond | Oc Disch | Nasal Disch | Temp | Cough | Cond | Oc Disch | Nasal Disch | Temp | Cough | Cond | Oc Disch | Nasal Disch | Temp | Cough | Cond | Oc Disch | Nasal Disch |
| C | 100.3 | - | - | - | - | 100.2 | - | - | - | - | 102.7 | - | - | - | - | 99.6 | - | - | - | - |
| C+1 | 99.6 | - | - | - | - | 99.6 | - | - | - | - | 100 | - | - | - | - | 100.4 | - | - | - | - |
| C+2 | 100.5 | - | - | - | - | 100.6 | - | - | - | - | 100.5 | - | - | - | - | 100.2 | - | - | - | - |
| C+3 | 100 | - | - | - | - | 98 | - | - | - | - | 104.8 | - | - | - | - | 99 | - | - | - | - |
| C+4 | 100.1 | - | - | - | - | 100.4 | - | - | - | - | 100.4 | - | - | - | - | 99 | - | - | - | - |
| C+5 | 99.8 | - | - | - | - | 99.8 | - | - | - | + | 100.2 | - | - | - | - | 100.6 | - | - | - | + |
| C+6 | 100.3 | - | - | - | - | 100.4 | - | - | - | - | 99 | - | - | - | - | 99.8 | - | - | - | - |
| C+7 | 99.9 | - | - | - | - | 99.6 | - | - | - | - | 100 | - | - | - | - | 100.1 | - | - | - | - |
| C+8 | 99.9 | - | - | - | - | 100 | - | - | - | + | 99.7 | - | - | - | - | 100.2 | - | - | - | - |
| C+9 | 98.8 | - | - | - | - | 101 | - | - | - | - | 99.9 | - | - | - | - | 100 | - | - | - | - |
| C+10 | 99.7 | - | - | - | - | 100 | - | - | - | - | 99.8 | - | - | - | - | 99.8 | - | - | - | - |
| C+11 | 99.8 | - | - | - | - | 100 | - | - | - | - | 99 | - | - | - | - | 99.9 | - | - | - | - |

| Day | Pony number | | | | | | | | | | | | | | | | | | | | | | | |
|------|-------------|-------|------|----------|-------------|-------|------|-------|------|----------|-------------|------|------|-------|------|----------|-------------|---|-------|-------|------|----------|-------------|---|
| | O4B | | | | | | C4D | | | | | | 40A | | | | | | A51 | | | | | |
| | Temp | Cough | Cond | Oc Disch | Nasal Disch | | Temp | Cough | Cond | Oc Disch | Nasal Disch | | Temp | Cough | Cond | Oc Disch | Nasal Disch | | Temp | Cough | Cond | Oc Disch | Nasal Disch | |
| C | 100.2 | - | - | - | - | 100 | - | - | - | - | - | 99.8 | - | - | - | - | - | - | 100.1 | - | - | - | - | - |
| C+1 | 100.1 | - | - | - | - | 100.1 | - | - | - | - | - | 100 | - | - | - | - | - | - | 100 | - | - | - | - | - |
| C+2 | 100.8 | - | - | - | - | 100.2 | - | - | - | - | - | 99 | - | - | - | - | - | - | 100 | - | - | - | - | - |
| C+3 | 101.9 | - | - | - | - | 100.6 | - | - | - | - | - | 100 | - | - | - | - | - | - | 100 | - | - | - | - | - |
| C+4 | 99.9 | - | - | - | - | 100.6 | - | - | - | - | - | 98 | - | - | - | - | - | - | 99 | - | - | - | - | - |
| C+5 | 99.4 | - | - | - | - | 100.2 | - | - | - | - | - | 99.4 | - | - | - | - | - | - | 99.6 | - | - | - | - | - |
| C+6 | 99.6 | - | - | - | - | 100.1 | - | - | - | - | - | 99.2 | - | - | - | - | - | - | 99.4 | - | - | - | - | - |
| C+7 | 99.6 | - | - | - | - | 100.2 | - | - | - | - | - | 99.6 | - | - | - | - | - | - | 99.8 | - | - | - | - | - |
| C+8 | 99.8 | - | - | - | - | 100.3 | - | - | - | - | - | 99.4 | - | - | - | - | - | - | 100.1 | - | - | - | - | - |
| C+9 | 99.6 | - | - | - | - | 99.9 | - | - | - | - | - | 99.4 | - | - | - | - | - | - | 100.1 | - | - | - | - | - |
| C+10 | 99.6 | - | - | - | - | 99.8 | - | - | - | - | - | 99.8 | - | - | - | - | - | - | 100.2 | - | - | - | - | - |
| C+11 | 100.4 | - | - | - | - | 99 | - | - | - | - | - | 99.2 | - | - | - | - | - | - | 100.1 | - | - | - | - | - |

extracts from 2 to 8 days after infection. The levels of NP reflected the amount of live virus which was shed, as measured by titration in hens eggs (figure 5.3.14)

Previously infected ponies

As expected, (Hannant *et al.*, 1988b) the two ponies which had previously been infected with influenza were resistant to challenge infection (table 5.3.2). These ponies did not show NP in nasopharyngeal swab extracts and did not shed live virus (figure 5.3.14).

Influenza specific ASC responses were detected in both ponies (figures 5.3.12 and 5.3.13), Increases in nasal wash and serum immunoglobulins were also observed in both ponies (figures 5.3.12 and 5.3.13).

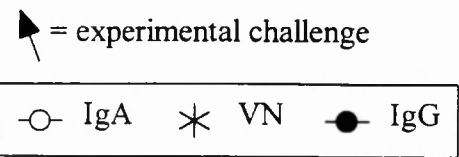
Vaccinates

All six intranasally boosted ponies were completely protected from influenza infection in that they did not exhibit any clinical signs (table 5.3.2) and did not show NP in nasal swab extracts or shed live virus (figure 5.3.14).

Figure 5.3.8 **Immunological responses of naive pony in response experimental infection.**

Data derived from a single pony: Pony V4

Figure 5.3.8 shows the nasopharyngeal and systemic antibody responses to an experimental infection.



Graph A, Influenza specific nasopharyngeal IgA and IgG on the left hand axis (expressed as arbitrary units (AU), as a function of total isotype) in relation to circulating influenza specific ASC on the right hand axis (expressed as a histogram showing numbers of ASC /10⁶ PBMC).



Graph B, Influenza specific nasopharyngeal IgA and IgG on the left hand axis(expressed as raw ELISA data, Ab.490nm) and virus neutralisation activity of the nasal washes on the right hand axis(expressed as a neutralisation index (NI), asterisk sign).

Graph C, Serum IgA and IgG on the left hand axis (expressed as raw ELISA data, Ab.490nm) and serum haemagglutination activity on the right hand axis (expressed as a histogram, log₂).

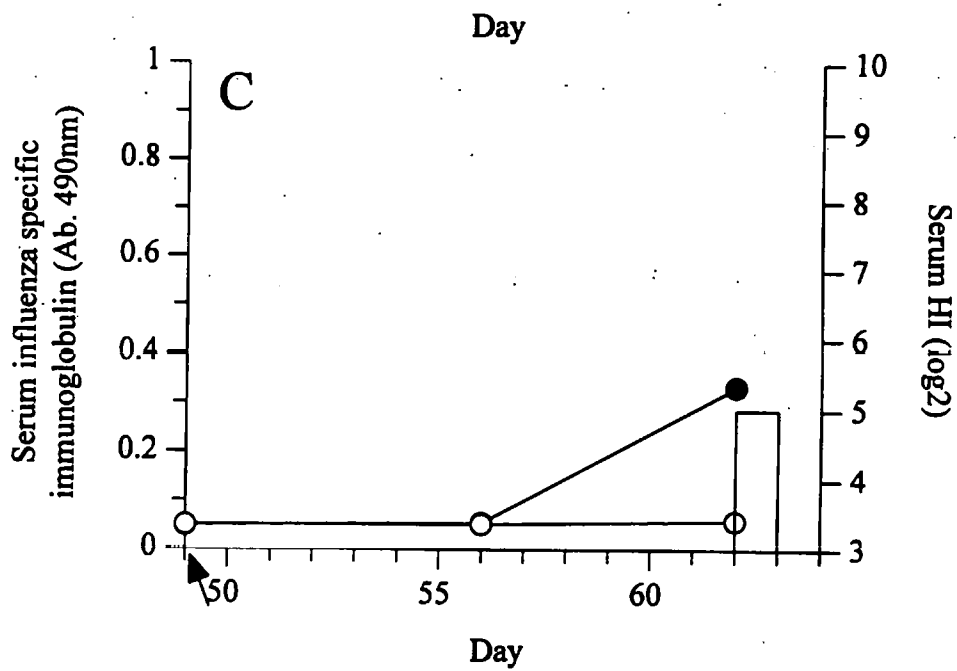
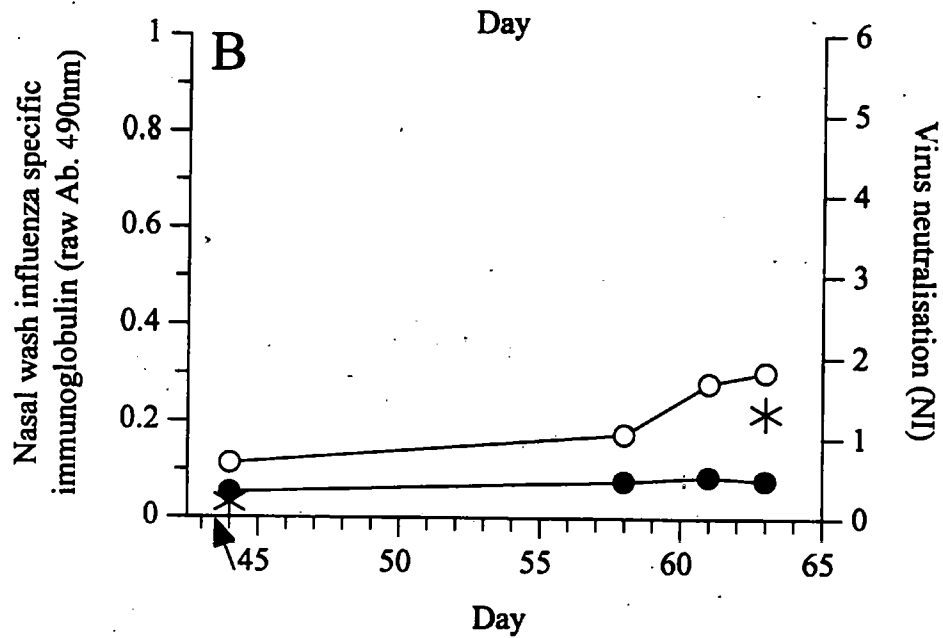
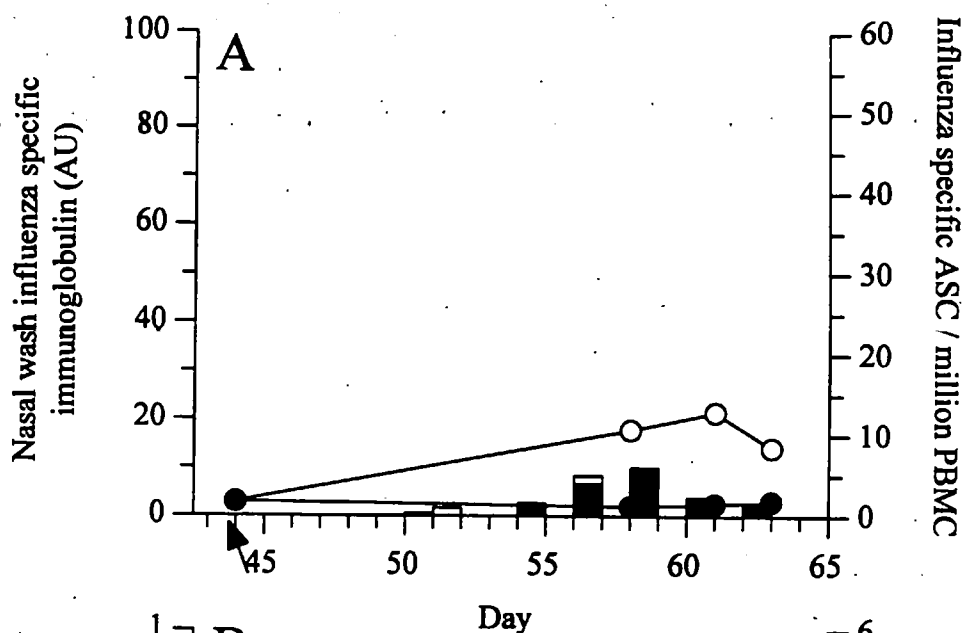
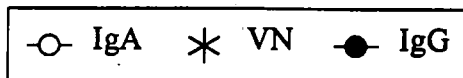


Figure 5.3.9 Immunological responses of naive pony in response experimental infection.

Data derived from a single pony: Pony V7

Figure 5.3.9 shows the nasopharyngeal and systemic antibody responses to an experimental infection.

▲ = experimental challenge



Graph A, Influenza specific nasopharyngeal IgA and IgG on the left hand axis (expressed as arbitrary units (AU), as a function of total isotype) in relation to circulating influenza specific ASC on the right hand axis (numbers of ASC /10⁶ PBMC).

□ IgG ASC ■ IgA ASC

Graph B, Influenza specific nasopharyngeal IgA and IgG on the left hand axis (expressed as raw ELISA data, Ab.490nm) and virus neutralisation activity of the nasal washes on the right hand axis (expressed as a neutralisation index (NI), asterisk sign).

Graph C, Serum IgA and IgG on the left hand axis (expressed as raw ELISA data, Ab.490nm) and serum haemagglutination activity on the right hand axis (expressed as a histogram, HI log₂).

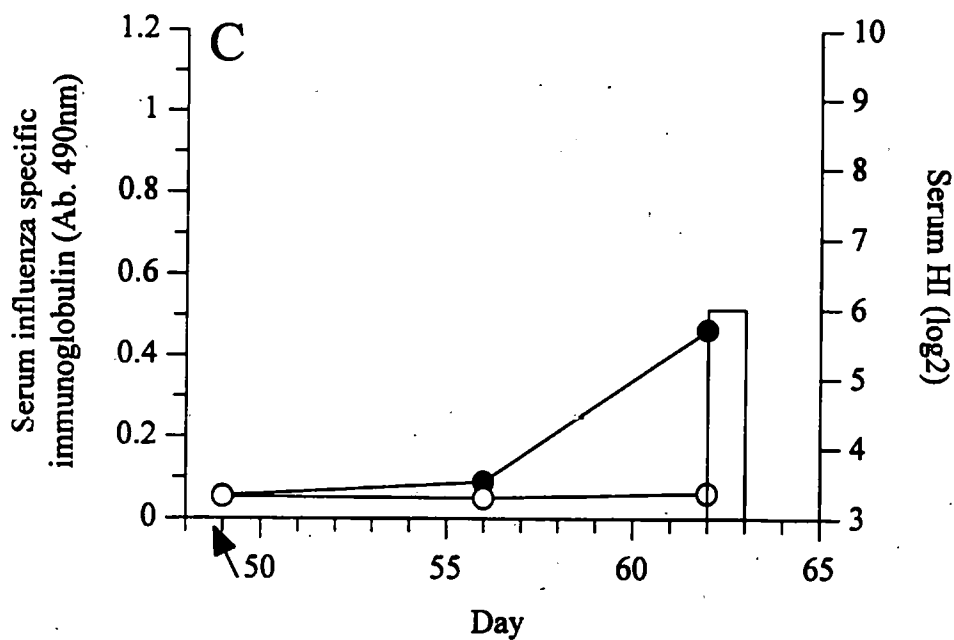
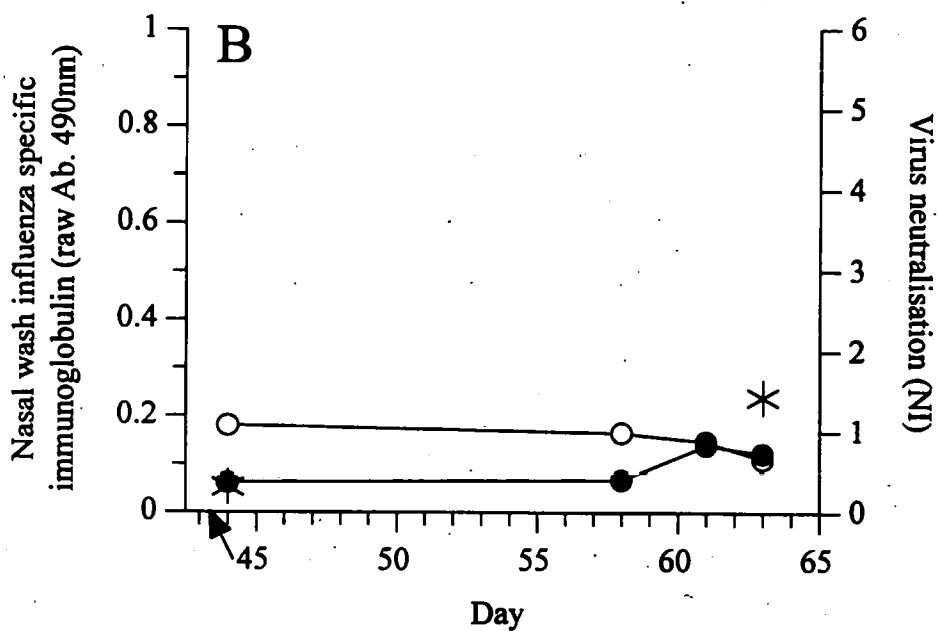
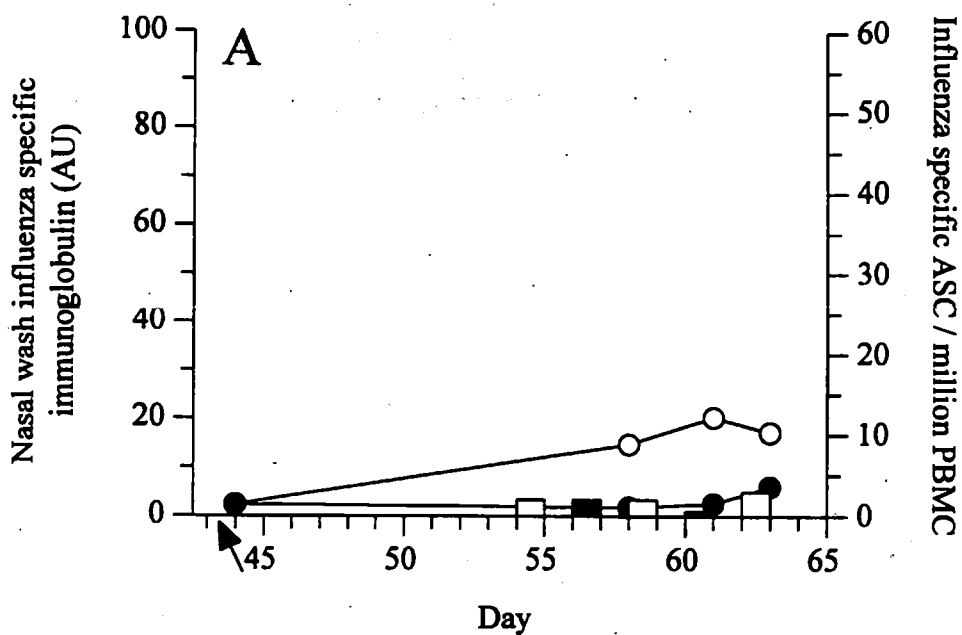
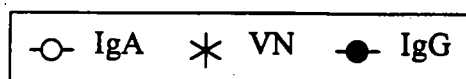


Figure 5.3.10 Immunological responses of naive pony in response experimental infection.

Data derived from a single pony: Pony V9

Figure 5.3.10 shows the nasopharyngeal and systemic antibody responses to an experimental infection.

▲ = experimental challenge



Graph A, Influenza specific nasopharyngeal IgA and IgG on the left hand axis (expressed as arbitrary units (AU), as a function of total isotype) in relation to circulating influenza specific ASC on the right hand axis (expressed as a histogram showing numbers of ASC /10⁶ PBMC).

□ IgG ASC ■ IgA ASC

Graph B, Influenza specific nasopharyngeal IgA and IgG on the left hand axis (expressed as raw ELISA data, Ab.490nm) and virus neutralisation activity of the nasal washes on the right hand axis (expressed as a neutralisation index (NI), asterisk sign).

Graph C, Serum IgA and IgG on the left hand axis (expressed as raw ELISA data, Ab.490nm) and serum haemagglutination activity on the right hand axis (expressed as a histogram, HI log₂).

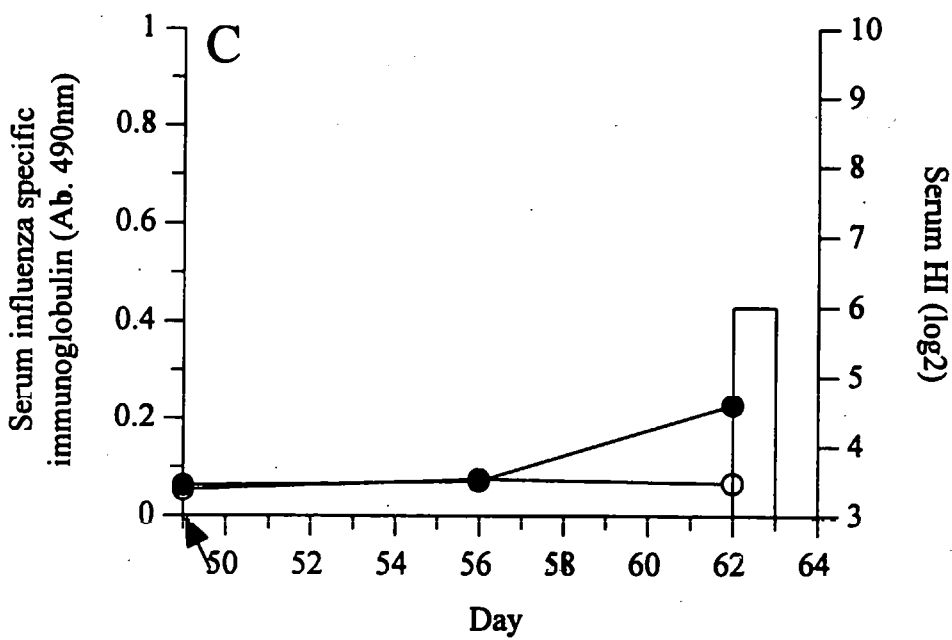
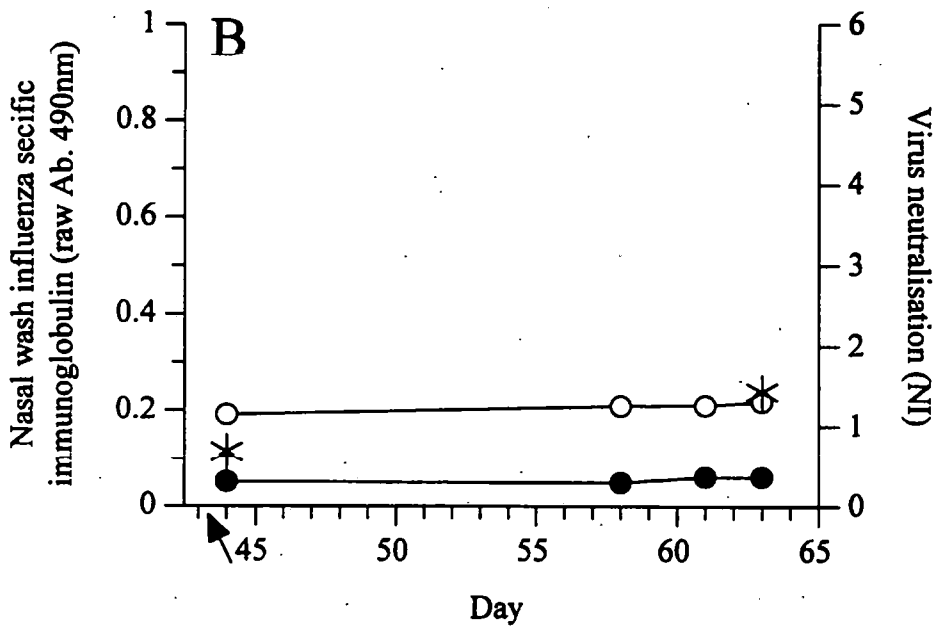
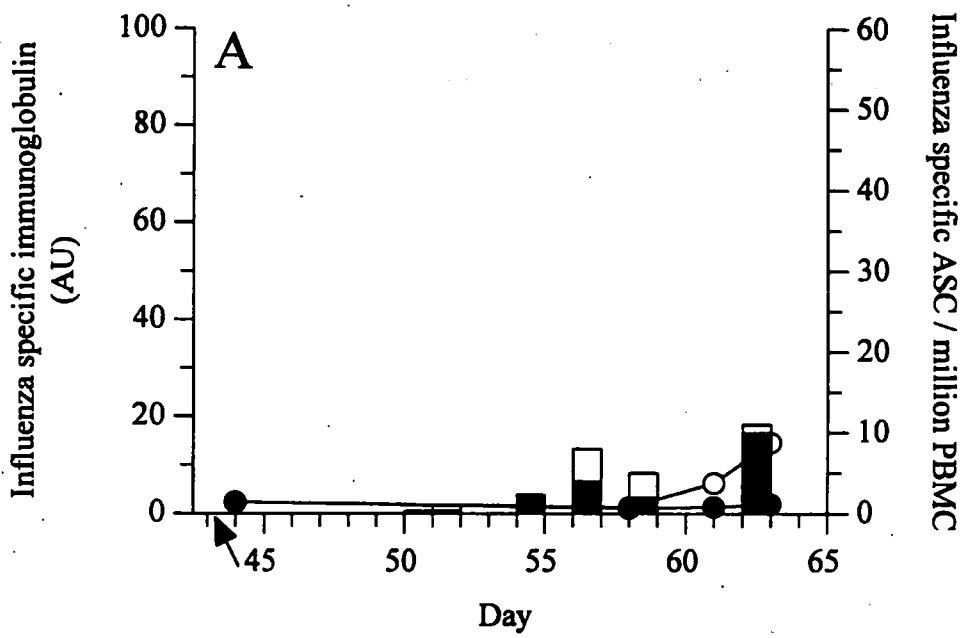
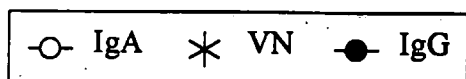


Figure 5.3.11 Immunological responses of naive pony in response experimental infection.

Data derived from a single pony: Pony V14

Figure 5.3.11 shows the nasopharyngeal and systemic antibody responses to an experimental infection.

▲ = experimental challenge



Graph A, Influenza specific nasopharyngeal IgA and IgG on the left hand axis (expressed as arbitrary units (AU), as a function of total isotype) in relation to circulating influenza specific ASC on the right hand axis (expressed as a histogram showing numbers of ASC /10⁶ PBMC).

□ IgG ASC ■ IgA ASC

Graph B, Influenza specific nasopharyngeal IgA and IgG on the left hand axis (expressed as raw ELISA data, Ab.490nm) and virus neutralisation activity of the nasal washes on the right hand axis (expressed as a neutralisation index (NI), asterisk sign).

Graph C, Serum IgA and IgG on the left hand axis (expressed as raw ELISA data, Ab.490nm) and serum haemagglutination activity on the right hand axis (expressed as a histogram, HI log₂).

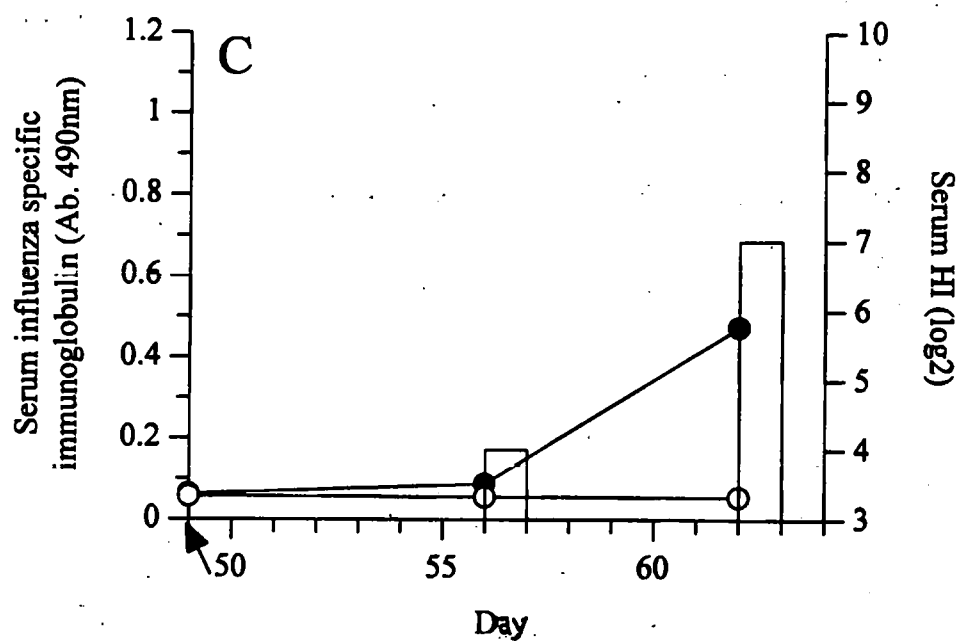
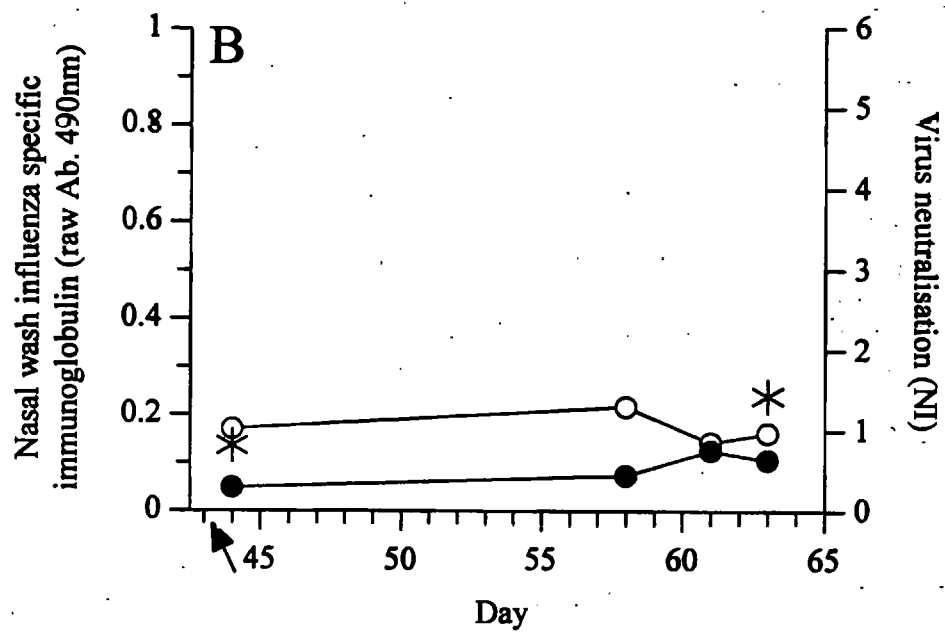
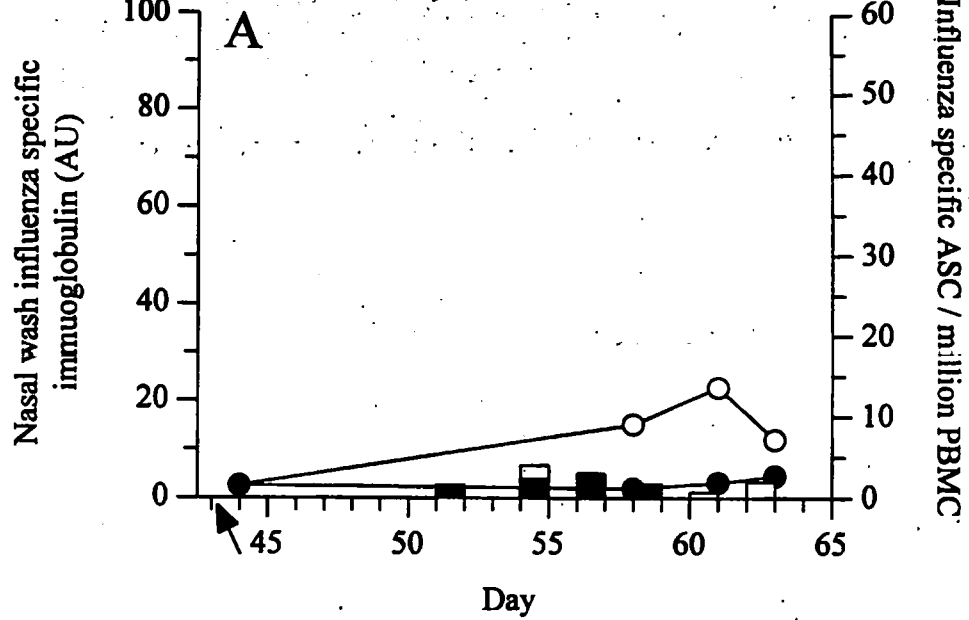
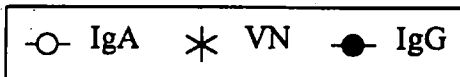


Figure 5.3.12 Immunological responses of a previously infected pony in response experimental infection.

Data derived from a single pony: Pony A51

Figure 5.3.12 shows the nasopharyngeal and systemic antibody responses to an experimental infection.

▲ = experimental challenge



Graph A, Influenza specific nasopharyngeal IgA and IgG on the left hand axis (expressed as arbitrary units (AU), as a function of total isotype) in relation to circulating influenza specific ASC on the right hand axis (expressed as a histogram showing numbers of ASC /10⁶ PBMC).

□ IgG ASC ■ IgA ASC

Graph B, Influenza specific nasopharyngeal IgA and IgG on the left hand axis(expressed as raw ELISA data, Ab.490nm) and virus neutralisation activity of the nasal washes on the right hand axis(expressed as a neutralisation index (NI), asterisk sign).

Graph C, Serum IgA and IgG on the left hand axis (expressed as raw ELISA data, Ab.490nm) and serum haemagglutination activity on the right hand axis (expressed as a histogram, HI log₂).

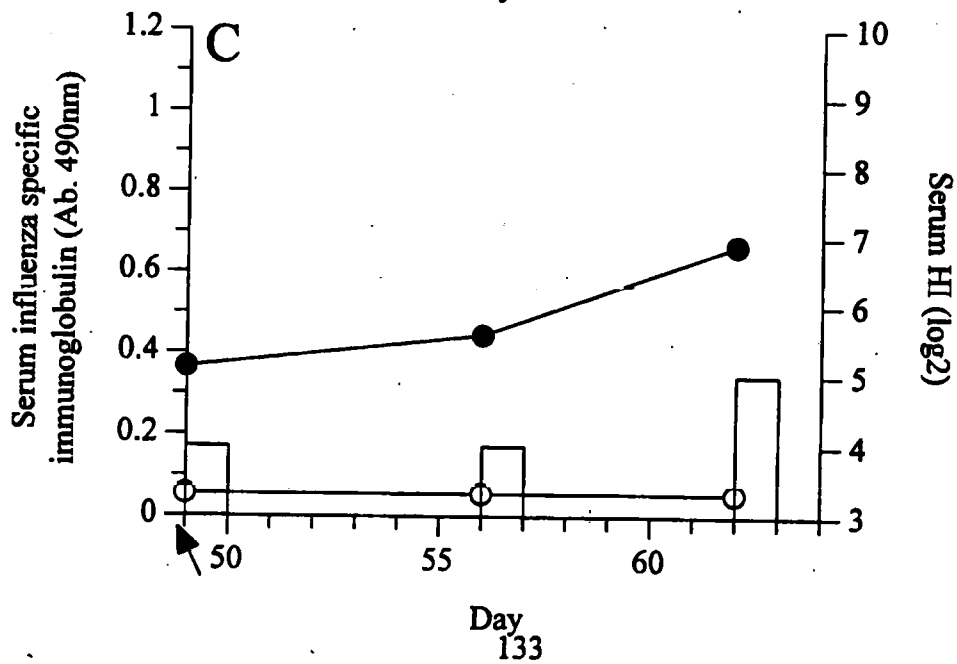
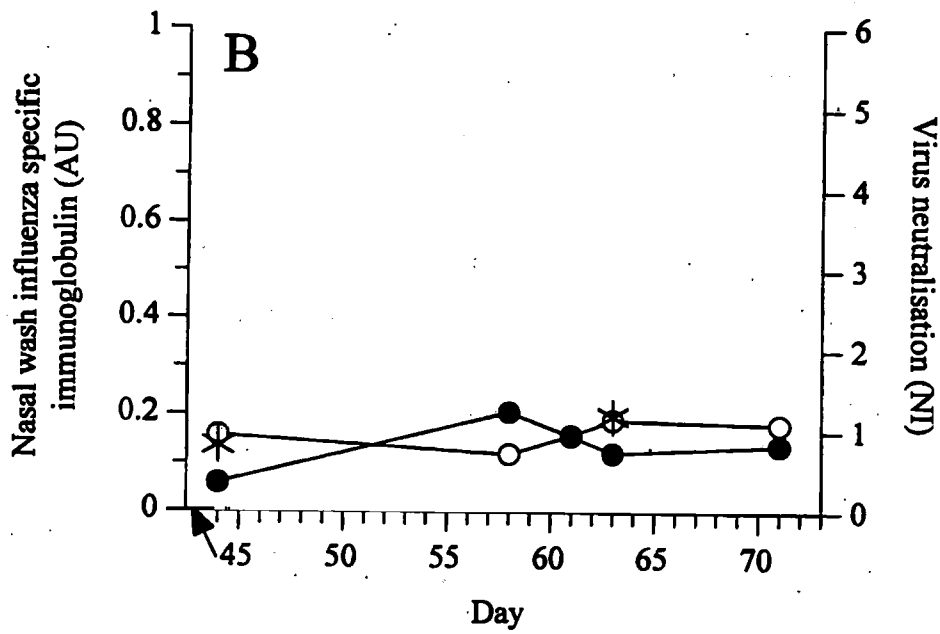
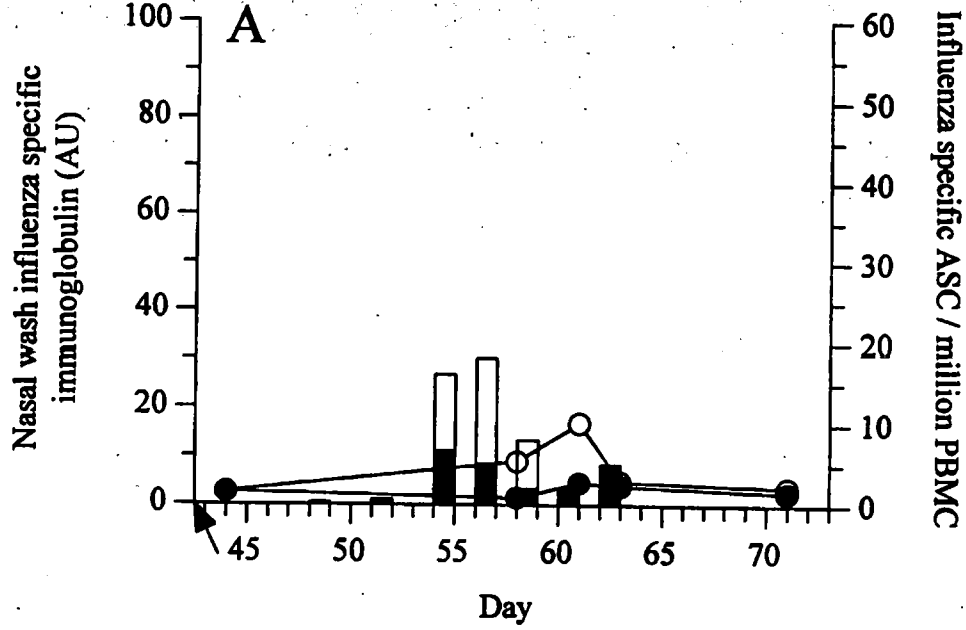
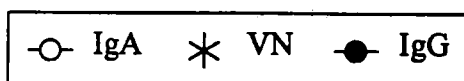


Figure 5.3.13 Immunological responses of a previously infected pony in response experimental infection.

Data derived from a single pony: Pony 40A

Figure 5.3.13 shows the nasopharyngeal and systemic antibody responses to an experimental infection.

▲ = experimental challenge



Graph A, Influenza specific nasopharyngeal IgA and IgG on the left hand axis (expressed as arbitrary units (AU), as a function of total isotype) in relation to circulating influenza specific ASC on the right hand axis (expressed as a histogram showing numbers of ASC /10⁶ PBMC).

□ IgG ASC ■ IgA ASC

Graph B, Influenza specific nasopharyngeal IgA and IgG on the left hand axis (expressed as raw ELISA data, Ab.490nm) and virus neutralisation activity of the nasal washes on the right hand axis (expressed as a neutralisation index (NI), asterisk sign).

Graph C, Serum IgA and IgG on the left hand axis (expressed as raw ELISA data, Ab.490nm) and serum haemagglutination activity on the right hand axis (expressed as a histogram, HI log₂).

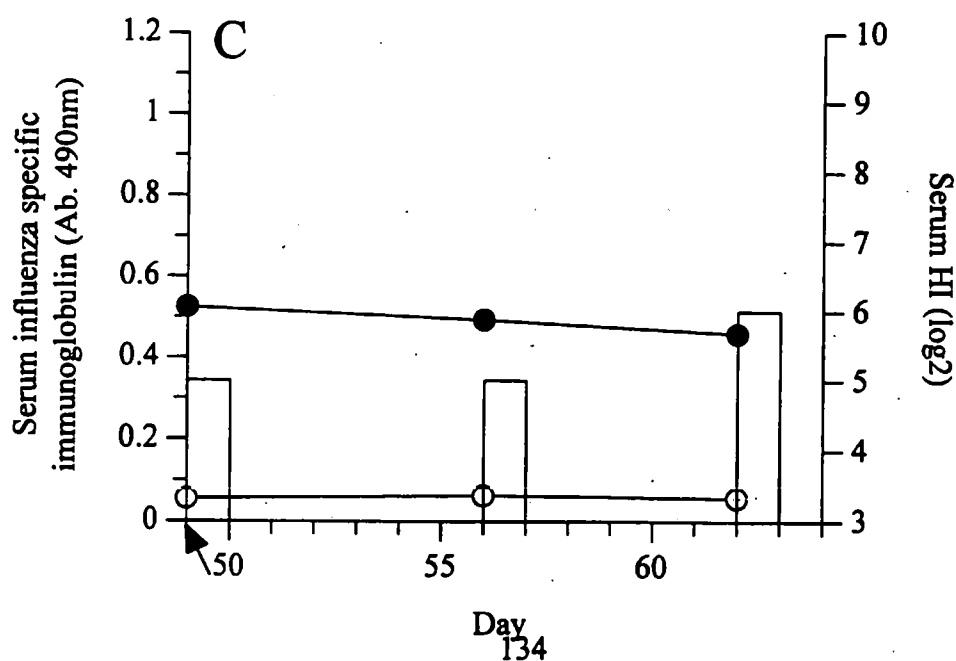
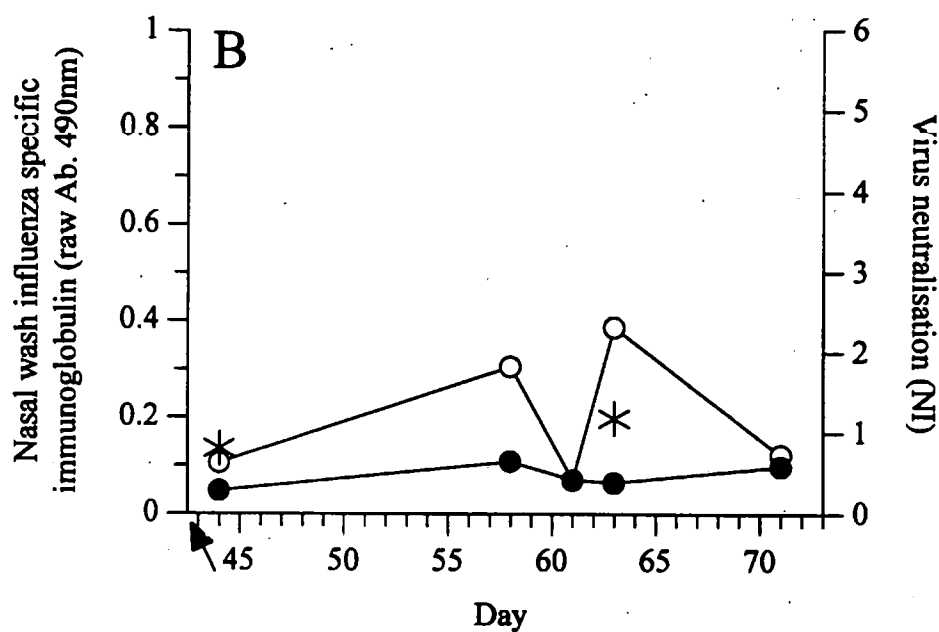
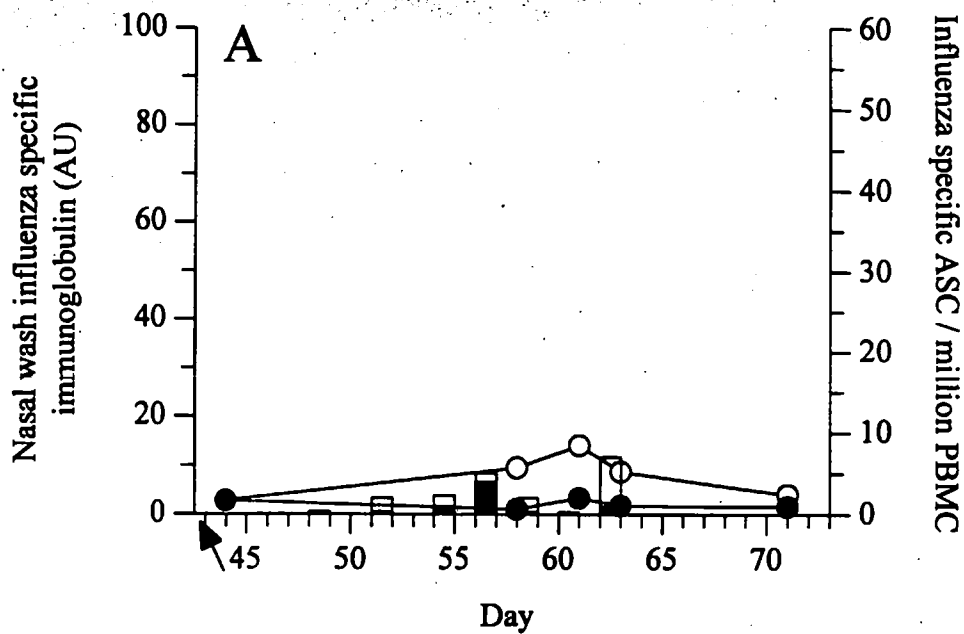
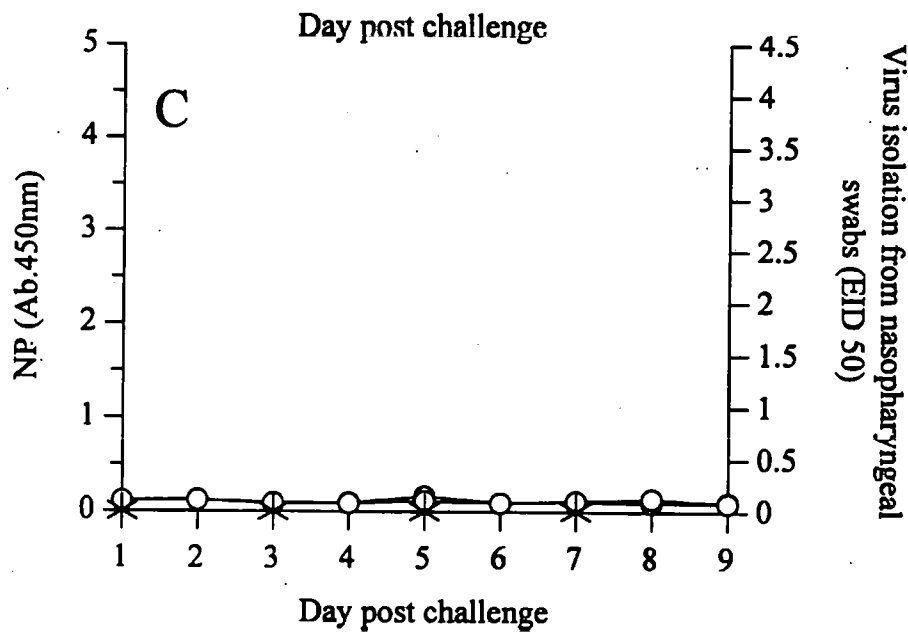
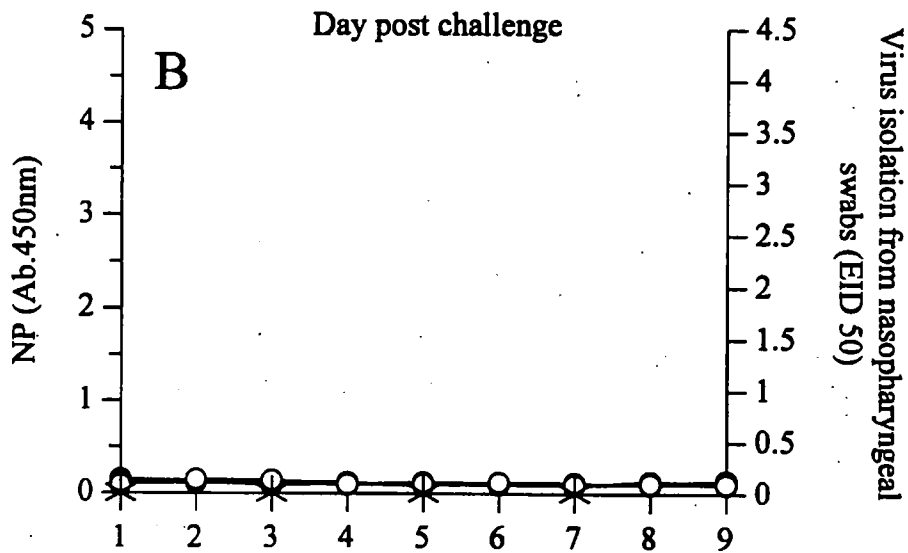
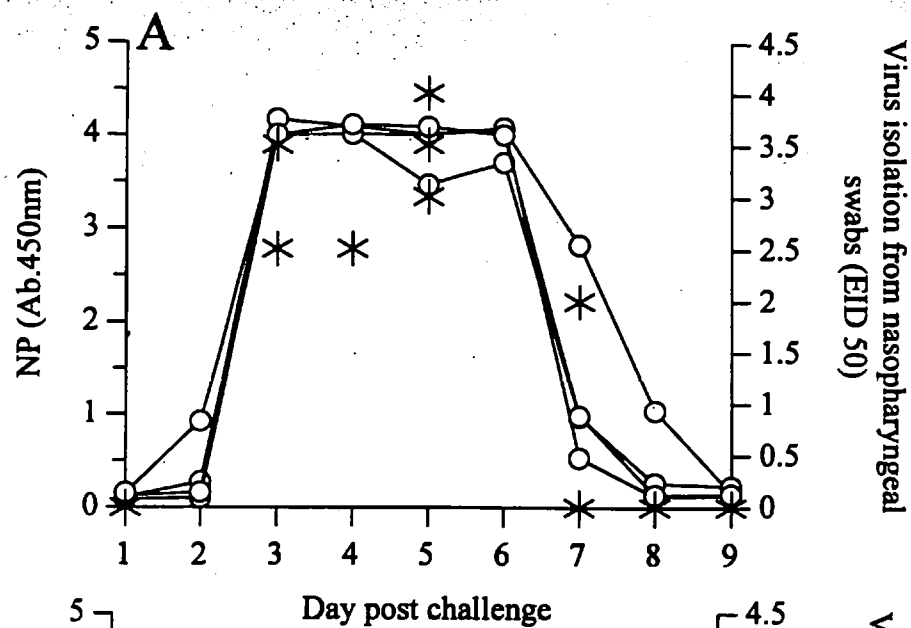


Figure 5.3.14 Nasal shedding of influenza virus after experimental challenge

Figure 5.3.14 shows influenza virus nucleoprotein as detected by ELISA (Ab.470nm) and virus isolation in fertile hens eggs (TCID₅₀). Graph A shows data from the negative control ponies (V4, V7, V9, and V14). Graph B shows data from the intranasally vaccinated ponies (F79, 90F, 1669, 6431, O4B, C4D), and graph C showing the positive controls (A51 and 40A) which had been infected six months previously.

○ NP ELISA ✱ VI in eggs



5.3.4 Summary of results

The table below (5.4.1) summarises the data for antibody and antibody secreting cells (ASC) at the time of infection, and the clinical/virological outcome of experimental challenge. Vaccinated ponies were F79, 90F, 1669, 6431, O4B, C4D, previously infected ponies were A51 and 40A, and unvaccinated control ponies were V4, V7, V9, V14.

Table 5.4.1 Summary of immunological and virological results

| Pony | Vac. (inf.) | Post Vaccination | | | | Post infection | | | | Infect (VI) |
|------|----------------|------------------|------------|-----------------------|--------------|---------------------|---------------------|-----------------------|--------------|----------------|
| | | IgA ASC | IgG ASC | Virus Neut (NI) | HI (log2) | IgA ASC (Day) | IgG ASC (Day) | Virus Neut (NI) | HI (log2) | |
| F79 | ✓ | 23 | 24 | 3.18 | 8 | 1 | 3 | 5.43 | 8 | ✗ (-) |
| 90F | ✓ | 25 | 18 | 2.43 | 9 | 1 | 5 | 2.81 | 9 | ✗ (-) |
| 1669 | ✓ | 47 | 30 | 4.06 | 10 | 2 | 5 | 5.43 | 10 | ✗ (-) |
| 6431 | ✓ | 7 | 12 | 1.06 | 7 | 2 | 2 | 2.31 | 7 | ✗ (-) |
| O4B | ✓ | 10 | 20 | 2.31 | 8 | 0 | 3 | 2.68 | 8 | ✗ (-) |
| C4D | ✓ | 6 | 25 | 0.68 | 6 | 0 | 1 | 1.43 | 6 | ✗ (-) |
| A51 | inf. | ND | ND | 0.81 | 5 | 7 | 18 | 1.18 | 5 | ✗ (-) |
| 40A | inf. | ND | ND | 0.81 | 5 | 4 | 5 | 1.18 | 6 | ✗ (-) |
| V4 | ✗ | ND | ND | 0.18 | <2 | 6 | 5 | 1.31 | 5 | ✓ (+) |
| V7 | ✗ | ND | ND | 0.18 | <2 | 2 | 3 | 1.43 | 6 | ✓ (+) |
| V9 | ✗ | ND | ND | 0.18 | <2 | 10 | 11 | 1.43 | 6 | ✓ (+) |
| V14 | ✗ | ND | ND | 0.18 | <2 | 3 | 4 | 1.43 | 7 | ✓ (+) |

footnotes:

Vac. = vaccinated i.n.(✓), negative control (✗), or positive control which has been infected previously (inf.)

Post Vaccination = Serological assays (NI and HI) assayed time point 2 days prior to challenge, ASC data was measured at various time points after the final intranasal boosting vaccination (data presented corresponds to the peak response observed amongst these time points).

Post Infection = Serological assays (NI and HI) assayed at a time point 14 days after infection, ASC data was measured at various time points after infection (data presented corresponds to the peak response observed amongst these time points).

IgA ASC and IgG ASC = Peak influenza specific ASC responses / 10^6 PBMC.

NI = Neutralisation index of influenza virus growth of the nasal washes.

HI = Haemagglutination inhibition titre of serum (log2).

ND = Not done.

Infect (VI) = Animal was infected: yes (✓) or no (✗) and virus was isolated from nasal swabs (VI): yes (+) or no (-).

The results presented above will be discussed fully in the Discussion section of this chapter (5.4), however, some *prima facie* observations may be drawn from the summarised data.

- All animals that had been vaccinated with the intramuscular prime/intranasal boost regime were protected from experimental infection.
- The virological outcome of the experimental infection in the vaccinated animal and the positive control group (A51 and 40A) was identical in that no virus could be detected in nasal swabs after infection.
- The serum HI titre in the vaccinated animal group was of a protective level prior to infection ($>5 \log_2$), this titre did not increase after infection.
- The level of nasal wash virus neutralisation activity in the samples taken from the vaccinated ponies before infection was greater than that seen in the nasal washes taken from the negative control ponies after infection (in 5 out of the 6 ponies).
- High levels of nasal wash virus neutralisation activity were not required to protect the positive control ponies from infection (A51 and 40A).

5.4 Discussion

Studies with conventional inactivated equine influenza vaccines have shown that protection from experimental infection is strongly correlated with the level of circulating antibody to haemagglutinin (Mumford *et al.*, 1983, 1988) and that after an influenza infection, clinical immunity in horses may last as long as 62 weeks (Hannant *et al.*, 1988b). This immunity, in contrast to the situation observed after vaccination, can be shown in animals with no detectable circulating influenza-specific antibody. Influenza-specific antibodies have also been detected in the mucosal secretions after an infection (Hannant *et al.*, 1988a, 1989a) and may be recalled very rapidly after new exposure (Hannant *et al.*, 1989a).

The studies described here with inactivated influenza antigens and CTB showed that in contrast with conventional systemic vaccines, intranasal vaccination of systemically primed ponies stimulates not only systemic antibody responses but also functional mucosal antibody responses, as measured by haemagglutination inhibition and virus neutralisation assays. Virus neutralising IgA occurs after natural infection and has been

implicated to be the major correlate of homo- and heterotypic immunity to influenza virus in other animals (Liew *et al.*, 1984; Asanuma *et al.*, 1995). The mucosal and systemic antibody responses induced in horses by influenza/CTB vaccination are very similar to those seen after equine influenza infection. However, currently there is no evidence for cellular immune responses induced by this type of mucosal vaccination of horses.

The intranasal vaccination of systemically primed horses with CTB and S89 induced sufficient mucosal virus neutralising IgA and systemic HI antibody to confer protection from experimental influenza infection (see 5.3.3.1). It is hypothesised that the success of the intranasal vaccination and ultimately protection from infection was dependent on systemic priming with influenza virus antigen. This statement is speculative as no controlled experiment was performed in which naive ponies were vaccinated with the nasopharyngeal applicator and a vaccine to which 2% holotoxin had been added; however it is in accord with some previously published studies (Barzu *et al.*, 1996). One can only speculate as to the reason behind this requirement for systemic priming, possibly the induction of naive T-cells to become memory T-cells may not be adequate within the local lymph node because of the unsuitable local cytokine environment or APC function. These features require further investigation.

It has been shown that animals vaccinated systemically with particular vaccine antigens may be protected clinically from heterotypic infection. However, these animals may still shed large amounts of live virus from their nasopharynx in the absence of clinical signs (Mumford *et al.*, 1997). For example, Equine influenza vaccines derived from North American influenza strains induced clinical protection when challenged with European strains of virus, however, these ponies shed significantly more live virus than the control ponies which had been vaccinated with influenza vaccines comprising European influenza virus strains (Mumford *et al.*, 1997). No speculation can be made as to the relative contribution of intranasal vaccine-induced mucosal antibody in protection from infection because of the high level of background circulating antibody which was also induced. This

HI antibody in all of the vaccinated ponies was of sufficient magnitude to confer protection in the absence of mucosal immunity. Mucosal influenza specific IgA has been implicated in heterotypic protection (Liew *et al.*, 1984; Asanuma *et al.*, 1995), and may therefore significantly improve protection from newly emergent or "foreign" viruses. Therefore, the advantages of mucosal vaccination may only be highlighted at a later date when experimental infections with heterotypic influenza virus are performed.

Influenza specific ASC were observed in the PBMC very rapidly after intranasal boosting. This response mimics the rapid recall responses seen after a second influenza infection (Hannant *et al.*, 1989a), and encourages the use of mucosal vaccination as an emergency measure to "blitz" vaccinate a stable yard in the face of an epidemic.

The inclusion of 2% CT holotoxin as a supplement to the vaccine mixture had a significant effect on the magnitude of the resultant immune response ($p < 0.05$). The numbers of CTB specific IgG ASC in the PBMC of vaccinated animals increased from a mean of 41 (mean ASC/ 10^6 PBMC, $n=3$) in animals that recieved CTB alone (mean age 6 months), to 258 (mean ASC/ 10^6 PBMC, $n=6$) in animals that recieved CTB plus 2% CT and influenza antigen, mean age 12 months). The control group described above is a composite group comprising animals which had been vaccinated using both the plastic pasteur pipette and the nasopharyngeal applicator. The effect of method of CTB administration on the resultant CTB specific ELISPOT responses was thought not to be significant, as previous experience showed no effect ($n = 2$).

These studies have shown conclusively that immunity may be induced in equids by intranasal vaccination regimes containing inactivated virus antigens. CTB specific immunological responses could be induced by intranasal priming and intranasal boosting. However, in order for bystander antigen specific responses to occur (in this case influenza virus), systemic priming by conventional intramuscular vaccination or previous infection was necessary before intranasal boosting.

Chapter 6

Discussion

Summary of findings

A fundamental requirement for the efficient functioning of a mucosal vaccine is a targeted vaccination procedure. The horse has well defined lymphoid areas within the nasopharynx which, because of its remote site, present problems of accessibility for vaccination. During this project I have developed an intranasal applicator, described in chapter 4. This apparatus allows a small quantity of vaccine to be delivered in a fine spray and coating a large area with vaccine, including all of the lymphoid structures. The nasopharyngeal applicator has also been utilised during the sampling of nasopharyngeal secretions. This improvement of an old technique has improved the reproducibility of the nasal washing procedure, in terms of reproducibility and overall yield.

Techniques have also been developed in order that data derived from nasal wash samples could be interpreted more reliably. It became clear during the course of these developments that day to day variations of secretions, and sampling efficiency, would make interpretation of raw ELISA data very difficult. The changes that occur after events, such as a vaccination or infection, in the physicochemical quality of the nasal washes make it necessary to express the antigen specific ELISA data as a function of the total amount of isotype specific immunoglobulin present in the nasal wash.

We have demonstrated previously that mucosal vaccination, or natural infection (Hannant *et al.*, 1988a and 1989a), can induce virus specific immunoglobulins in the nasal secretions in the horse. This study, however, has been the first to describe functional activity of those nasopharyngeal antibodies. The nasal washes taken after intranasal vaccination show haemagglutination inhibition and virus neutralisation (VN) activity in

tissue culture. The VN activity of the nasal washes correlated with the levels of virus specific IgA present in the nasal wash.

This study has been the first investigation of the mucosal immune system in the horse. It has made the first tentative confirmation, by the demonstration of trafficking B cells after mucosal vaccination, of the existence of a local mucosal immune system in the horse. It is proposed that the antigen specific IgA antibody secreting cells (ASC) are trafficking in a targeted manner to the mucosal lamina propria within the upper respiratory tract. The antigen specific IgG ASC do not migrate preferentially to mucosal sites such as the upper respiratory tract. However, it may be that the non-appearance of influenza specific IgG in the nasopharynx may purely be because of a lack of IgG transport from the LP into the airway lumen.

This thesis is the first description of a mucosal vaccine in the horse. The vaccine formulated with heat inactivated influenza antigen and cholera toxin B chain, was successful in inducing systemic and nasopharyngeal anti-influenza immune responses. These immunological responses were sufficient to protect horses fully from an experimental influenza infection. This study is one of very few that have been previously published (Israel *et al.*, 1992; Hoshi *et al.*, 1995) in which a mucosal vaccination confers protection from an important species specific pathogen within its natural host.

Future use and development of mucosal vaccines

To achieve commercial success the vaccine of the future will have to fulfil several requirements for the modern world. Each vaccine will have to work with great efficacy, be easily and painlessly administered by non-professionally qualified personnel, and remain

stable enough to survive long periods of transport. Veterinary vaccine manufacturers are already looking into the possibility of moving away from "needle" based vaccines, in order to make the inoculation itself more acceptable to both patient and owner (R.Jacobs, personal communication). Mucosal vaccines hold many advantages in a veterinary market. The ease of application and the possibility of mass vaccination, through water troughs or vaccine nebulised into the air, make it very attractive to veterinarians treating large numbers of communal animals. This approach may be less applicable to species like the horse when non-living vaccines are used which may require too high an antigen dose or too frequent boosting. As such, mucosal vaccines are receiving much attention from researchers and pharmaceutical company marketing departments alike.

No speculations have been made as to the relative contribution of mucosal antibodies to the protection of the ponies from experimental infection. Israel *et al.* (1992) attempted to address this question in cattle by electron microscopy on the epithelium of cattle challenged with bovine herpes virus-1 (BHV-1). This experiment demonstrated the failure of virions to bind onto and penetrate the epithelium of cattle which had been intranasally vaccinated with BHV-1 and CTB, and experimentally challenged with infectious BHV-1. The anti-influenza role of mucosal antibodies could be shown in the future, in a less invasive fashion, by the challenge of mucosally and systemically vaccinated groups with heterologous virus. With the putative benefits of mucosal antibody in the heterotypic protection of influenza virus, the mucosally vaccinated animals may be distinguishable from the systemically vaccinated ponies by virtue of the fact that a much reduced nasal shedding of live virus would be observed after experimental infection.

In general, very little is currently known about the interaction of antigen presenting cells (APC) and T-cells in the mucosal immune system. Still less is known about APC in the horse. Dendritic cells (DC) have been identified as major APCs in the mucosal immune

system (Havenith *et al.*, 1993; Holt *et al.*, 1994; Xia *et al.*, 1995), and have recently been the target of much research in the equid (Seidec *et al.*, 1995). Investigations to define the role of DCs is therefore an interesting direction for future research to take. These experiments could include using antigen-pulsed DCs as experimental vaccines, and the use of efferent DCs after a mucosal vaccination to stimulate T-cell activity *in vitro*. Such experiments would confirm the role of DCs in the induction of a mucosal immune response and allow the construction of more intelligently designed vaccines.

The ELISPOT assay which has now been developed is a powerful tool for the investigation of immune responses. In other species, B-cells observed after a mucosal vaccination traffic to distant mucosal effector sites because of their expression of integrins that interact specifically with ligands expressed on endothelia at mucosal sites. In the human the integrin $\alpha 4\beta 7$ expressed on the lymphocyte specifically binds to the MADCAM-1 molecule present on the high endothelial venule (HEV) of mucosal epithelia (Kilshaw and Murant, 1991; Hu *et al.*, 1992; Berlin *et al.*, 1993; Erle *et al.*, 1994; Hamann *et al.*, 1994; Viney *et al.*, 1996). The expression of such integrins and addressins should be investigated in the equid. If parallel systems can be established, any immune response to systemic or mucosal vaccination could be investigated. This approach would be useful if systemic routes of vaccination were to be developed in order to induce mucosal antibody (Daynes *et al.*, 1996).

This thesis does not investigate the induction of antigen specific T-cell responses by mucosal vaccination. Recently, CTB combined with influenza virus nucleoprotein (NP) has been shown to induce cellular responses which have an important role in the clearance of virus from infected mice (Tamura *et al.*, 1996). The intranasal vaccination of these mice with recombinant NP and CTB induced systemic delayed-type hypersensitivity (DTH) reactions, increased production of INF- γ in response to virus *in vitro*, increased

numbers of nasal CD4 +ve T-cells after challenge, but not NP-specific cytotoxic lymphocytes (CTL). Protection from other equine viruses, such as equine herpes virus-1 (EHV-1), is thought to require a strong CTL response to infection. If CTB, as an adjuvant does not induce CTL responses, more intelligently designed vaccines which will induce endogenous presentation of antigen would therefore have to be investigated.

In the future it will be necessary to design each new mucosal vaccine in accordance with the identified correlates of immunity. Each disease will have to be treated individually according to the types of protective immune response involved. New adjuvants and delivery vectors, as well as DNA technology, need to be investigated in the horse. The use of vectors such as liposomes will allow great flexibility for the induction of mucosal immune responses. The particulate nature of liposomes will increase the uptake of antigen by cells such as DCs, while cytokines such as GM-CSF could be encapsulated and stimulate the DCs further to induce both cellular and humoral immunity. Liposomes can also be used to boost responses of peptide vaccines by better presentation of the peptide for endogenous processing. In this manner the vaccine could be further targeted, and designed in a manner which is optimal for each given pathogen.

Mucosal vaccination has great potential for use in the future. It may induce systemic immune responses at least as well as conventional vaccination, and also confers the advantages of mucosal immune responses. The putative requirement for systemic priming may not necessarily be a huge drawback in the human field, as an intranasal vaccine would be much more acceptable than repetitive needle based boosters. The advantages of mucosal vaccines might include mucosal virus neutralising antibodies and intraepithelial CTL, which together can prevent infection at the site of initial contact. This may be particularly important where pathogens quickly pass the epithelial barrier and set up immune suppression and latency.

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List of Abbreviations

| | |
|---------|--|
| AM | Alveolar Macrophage |
| APC | Antigen Presenting Cell |
| ASC | Antibody Secreting Cells |
| BAL | Broncho-alveolar Lavage |
| BALT | Bronchus Associated Lymphoid Tissue |
| BBS | Borate Buffered Saline |
| BCIP | 5-bromo-4-chloro-indolyl phosphate |
| BHV | Bovine Herpes Virus |
| BSA | Bovine Serum Albumin |
| cAMP | Cyclic AMP |
| CMIS | Common Mucosal Immune System |
| CT | Cholera Toxin |
| CTA | Cholera Toxin A chain |
| CTB | Cholera Toxin B chain |
| CTL | Cytotoxic T-Lymphocyte |
| DC | Dendritic Cell |
| DTT | Dithiothreitol |
| EHV | Equine Herpes Virus |
| ELISA | Enzyme-linked Immunosorbent Assay |
| ELISPOT | Enzyme-linked Immunosorbent Spot forming assay |
| ESA | Equine Serum Albumin |
| FCS | Fetal Calf Serum |
| GALT | Gut Associated Lymphoid Tissue |
| Glut | Glutaraldehyde |
| GM1 | Monosialoganglioside GM1 |
| HA | Haemagglutination |
| HAU | Haemagglutination Unit |
| HEL | Hen Egg Lysozyme |
| HEV | High Endothelial Venule |
| HI | Haemagglutination Inhibition |
| HSV | Herpes Simplex Virus |
| IEL | Intra-epithelial Lymphocyte |
| ISCOM | Immune Stimulating Complex |
| LAK | Lymphokine-activated Killer cell |
| LP | Lamina Propria |
| LPL | Lamina Propria Lymphocyte |
| LTB | E.Coli heat-labile enterotoxin B sub-unit |
| MALT | Mucosae Associated Lymphoid Tissue |
| MDCK | Madin Derby Canine Kidney cells |
| MHC | Major Histocompatibility Complex |
| NALT | Nasopharyngeal Associated Lymphoid Tissue |
| NI | Virus Neutralisation Index |
| NK | Natural Killer Cell |

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|------|--|
| OPD | O-phenyldiamine |
| OVA | Ovalbumin |
| PBMC | Peripheral Blood Mononuclear Cells |
| PBS | Phosphate Buffered Saline |
| pIgR | Polymeric Immunoglobulin Receptor |
| PP | Peyers' Patch |
| RT | Respiratory Tract |
| S89 | A/Equi/2/Sussex/89 Influenza |
| sIgA | Secretory IgA |
| SIV | Simian Immunodeficiency Virus |
| SPDP | 3-(2-pyridyldithio) propionic acid succinimide ester |
| TcR | T-Cell Receptor |
| Th1 | T-helper class 1 |
| Th2 | T-helper class 2 |
| URT | Upper Respiratory Tract |
| VI | Virus isolation |
| VN | Virus neutralisation |
| VTM | Virus Transport Medium |